EVOLUTION OF PLANT DEFENSES AND HERBIVORE HOST SELECTION IN THE NEOTROPICAL GENUS OF TREES *INGA* (FABACEAE)

by

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ABSTRACT

Because plants and their insect enemies are strikingly species-rich groups, understanding their interactions has been a key issue in ecology and evolution. The arms race between plants and herbivores is considered the driver of diversification in both groups. However, we have a poor understanding of how these processes lead to divergence and speciation. This dissertation research tests key theories that relate plant-insect interactions with diversification and coevolution in both groups of organisms.

In the first part, I assess the utility and contrast the predictions of two theories aimed to explain the patterns of defense investment across species: The Apparency Theory and the Resource Availability Hypothesis. My results provide strong support for the predictions of the Resource Availability Hypothesis. In particular, the evolution of defenses appears to be related to interspecific differences in inherent growth rate rather than to a species' predictability to herbivores. The theory appears robust across latitude and ontogeny suggesting that it has served as a valid framework for investigating the patterns of plant defenses and that its applicability is quite general.

In the second part, I focus on how herbivores may drive the evolution of plant defenses, how plant defenses shape herbivore host choice and how plant-herbivore interactions might influence community composition and diversity focusing on the Neotropical genus of trees *Inga* (Fabaceae). I characterize the entire suite of anti-herbivore defenses and also quantify the diversity and abundance of leaf-feeders

associated with *Inga*. With the use of phylogenies for both plants and herbivores, I discriminate among possible macroevolutionary hypothesis of host use and plant defense evolution. Contrary to much coevolutionary theory, my results show that closely related *Inga* species are more divergent in anti-herbivore defenses than in non-defense traits, and that the evolution of host use in herbivorous insects is more conserved with respect to host defenses rather than to host phylogeny. Together, these results suggest that defenses evolve rapidly and that traits related to host choice evolve more slowly. Specifically, although divergence in herbivores might not be driven by their interactions with plants, herbivores may be an important factor driving the divergence among plant species.

This dissertation is dedicated to my daughter Emilia and my husband David

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CHAPTER 1

INTRODUCTION

Background

A primary goal in ecology and evolution is to understand the origin and maintenance of biodiversity. Major subjects of such inquiries are the plants and their insect herbivores for together they account for more than half of the macroscopic diversity on land. Theory has long predicted that the evolution of plant anti-herbivore defenses and insect counter-adaptations are the drivers of such a great diversity (Ehrlich & Raven 1964). However, we have a poor understanding of how these processes might lead to divergence and speciation. Consequently, for my dissertation research, I focus on a genus of trees and its associated herbivores to test key theories that relate plant-insect interactions with diversification and coevolution in both groups of organisms.

Herbivores exert major selective pressures on plants (Agrawal *et al.* 2012). In response, plants have developed a variety of defensive traits, which are particularly prevalent in young, expanding leaves (Coley & Aide 1991; Kursar & Coley 2003; Brenes-Arguedas *et al.* 2006). These include physical structures (e.g. hairs, thorns), secondary chemical compounds (e.g., phenolics, saponins, non-protein amino acids), nutritional quality, and phenological escape. These defensive traits are considered direct

defenses because they act directly on the herbivore. Plants have also evolved partnerships with species from the third trophic level. These are termed indirect defenses, and involve many different mechanisms. For example, plants may provide food to predators of insect herbivores (e.g., extrafloral nectar) or produce volatile signals to attract parasitoids of herbivores. However, herbivores have also developed a series of physiological, morphological and behavioral counter-adaptations to exploit their host plants. Thus, plants and insects appear to be reciprocally inducing evolutionary change between each other.

For many years the study of plant-insect interactions focused primarily on the role of secondary metabolites in plants and on the study of the costs and benefits of defenses. Once the significance of secondary chemicals in defense and in host selection was established (Dethier 1954; Frankel 1959), interest shifted to explaining why the amount and type of defense differed considerable between species. For example, Feeny (1976) proposed that differences in defenses between species are related to a species' predictability to herbivores; Coley *et al.* (1985) suggested that the evolution of defenses is related to interspecific differences in inherent growth rate. These theories stimulated a multitude of studies and established the basic conceptual framework linking defense and herbivory.

However, it was Ehrlich & Raven (1964) who incorporated a macroevolutionary framework into the study of plant-insect interactions. In their seminal paper, they considered the reciprocal nature of the adaptive responses between plants and herbivores and introduced the idea of coevolution between plants and butterflies, a concept that has

dominated our understanding of the interactions between plants and insects for the last 50 years.

According to Ehrlich & Raven's paradigm there is taxonomic conservatism in the expression of defenses in plants and in the use of hosts in insect herbivores. They suggested that this results from an ongoing coevolutionary arms race between plants and enemies. Specifically, this theory predicts that, after the evolution of a key innovation or "a new defense" in response to herbivore pressure, a plant species would be able to escape most herbivores and radiate into a clade of chemically similar plants. Thus, closely related plants would have similar defenses. Similarly, selection would favor counter-adaptations from herbivores to the novel plant defense and ultimately adaptive radiation of the herbivores onto a set of closely related plants. Hence, related insects should use related plants (Ehrlich & Raven 1964). Because of the defense and counter-defense coevolution pattern, clades of plants and insect herbivores are expected to mirror each other in their patterning of speciation events (e.g., topology of their phylogenies). Ehrlich & Raven suggested that these historical processes of radiation in both organisms may explain a substantial fraction of plant and insect diversities.

Ever since its publication, the coevolutionary theory of arms race was rapidly accepted and profoundly shaped the field. Evolutionary conservatism in plant-insect association became the new paradigm. Nevertheless, although explicit tests are few, they have found contradictory results. Examinations of the phylogenetic structure of plant defenses have revealed that close relatives tend to be dissimilar in defenses (Agrawal & Fishbein 2006; Bursera 1997; Becerra *et al.* 2009; Kursar *et al.* 2009; Sedio 2013). Furthermore, host shifts among closely related herbivores are more strongly correlated

with the chemistry of the new hosts than with their phylogenetic relationships (Becerra 1997; Becerra & Venable 1999; Berenbaum 2001; Wahlberg 2001), and phylogenies of insects rarely match those of their hosts (Futuyma & Agrawal 2009; Thompson 1994). Thus, the wide acceptance of a strong phylogenetic conservatism for defenses in plants and, hence, for host use in herbivores may not be warranted, suggesting that these ideas need refining and more rigorous testing.

Here, I examine macroevolutionary patterns of defense evolution and the contribution of plant-insect interactions to divergence in defenses among species and to community assembly. First, by means of a meta-analysis, I assess the utility of two of the most influential theories aimed to explain the patterns of defense investment across species. Second, I perform a complete characterization of plant functional traits and evolutionary relationships for plants and insect herbivores and determine how they fit into the arms race scenario. For this, I use *Inga,* a genus of trees and its associated herbivores in the Peruvian Amazon, a habitat where the diversity of plants and invertebrates is among the highest in the terrestrial world and where the arms race may be particularly pronounced.

Study system: The genus of trees *Inga* and its herbivores.

I focus my study on the speciose (>300 species), ecologically important, and abundant Neotropical tree genus, *Inga* (Fabaceae). This high diversity appears to be the result of a recent, explosive radiation within the last 4-10 million years (Richardson *et al.* 2001). At my study site in Peru, Los Amigos, and elsewhere, the genus *Inga* constitutes one of the most abundant and diverse genera (N. Pitman *unpublished data*), with more

than 40 species occurring in 25 ha and 6% of the stems (Valencia *et al.* 2004). If not the highest, *Inga* certainly is among those genera with the greatest number of congeneric species at a given site.

The study of defenses in *Inga* is exceptional in that in a single genus we can find multiple resistance traits. *Inga* produces a variety of secondary metabolites including non-protein amino acids, flavonoids, flavan-3-ols, saponins and amines (Lokvam *et al.* 2004; Lokvam & Kursar 2005; Brenes-Arguedas *et al.* 2006; Lokvam *et al.* 2007). The content of nitrogen in the leaf, a key nutritional trait for herbivores, varies twofold during the development of the young leaf (Kursar & Coley 2003). Also, *Inga* is characterized by the presence of nectaries on leaves that produce nectar and attract ant defenders (Koptur 1984). Some species have other physical (trichomes) and phenological (synchrony in leaf production) defenses as well (Coley *et al.* 2005).

Inga is associated with several groups of herbivores including Coleoptera, Orthoptera, phloem-feeding Coreidae, Diptera, sawflies, Phasmida and Lepidoptera. However, the group causing the most damage to leaves of *Inga* is Lepidoptera (Kursar *et al.* 2006). For this study I focused only on lepidopteran herbivores due to their importance.

Chapter summaries

Revisiting the Resource Availability Hypothesis (Chapter 2)

Among theories of plant defense, the Apparency Theory (Feeny 1976) and the Resource Availability Hypothesis (Coley, Bryant & Chapin 1985) stand out as two of the most influential plant defense theories in the last few decades. These theories have aimed

to explain patterns of defense investment and the selective pressures that have led to the variety of defensive strategies across species. The Resource Availability Hypothesis relates the evolution of defenses to interspecific differences in inherent growth rate, whereas Apparency Theory assumes that defenses are related to a species' predictability to herbivores. Although the theories have different assumptions regarding the reasons leading to defense differences, some of the predictions are similar. For example, both theories agree that long-lived, slow-growing species (apparent species) should invest more in defenses than short-lived, fast-growing species (unapparent species). However, a fundamental difference between the theories is their contrasting predictions for the amount of herbivory. The Resource Availability Hypothesis predicts that fast-growing species should suffer greater herbivore damage, while Apparency Theory predicts similar losses for apparent and unapparent species. Predictions from the Apparency Theory and the Resource Availability Hypothesis have been repeatedly tested. Overall, the evidence provides mixed support: some of the results are consistent with the predictions of the hypotheses whereas others not. In an early review (Stamp 2003), the assumptions and evidence for all theories of plant defense were summarized. However, to our knowledge, no attempt to revise quantitatively the utility of these two theories has been considered. For this manuscript, we provide a historical review of both theories, present evidence that shaped their development and, by means of a meta-analysis, contrast their predictions with results from many studies. Our results provide strong support for the predictions of the Resource Availability Hypothesis. In particular, species adapted to resource-poor environments 1) grow inherently more slowly, 2) invest more in constitutive defenses, and 3) support lower herbivory than species from more productive habitats. We also

found that the application of this theory appears robust across latitude and ontogeny, as the magnitude of the effect sizes for most of the predictions did not vary significantly between ecosystems nor across ontogenetic stages. Our data also showed that variation in growth rate among species better explains the differences in herbivory than variation in apparency, suggesting that the evolution of different defensive strategies across species is resource, rather than herbivore-driven. Therefore, we suggest that the Resource Availability Hypothesis is very broadly applicable for understanding the evolution of anti-herbivore defenses on timescales that are long and that probably correspond to the origin of clades.

Defense evolution, herbivore host selection and plant-insect evolutionary relationships in the species-rich Neotropical genus of trees *Inga*

(Chapters 3 & 4)

The remainder of my dissertation addresses the evolution of anti-herbivore defenses on timescales that correspond to the evolution of species. The arms race between plants and insect herbivores has been invoked as one of the main mechanisms driving trait divergence and coevolution for both groups (Becerra 1997; Thompson 1998; Becerra 2009; Futuyma & Agrawal 2009; Kursar *et al.* 2009; Agrawal 2012). Specifically, this theory proposes that adaptations between both organisms are reciprocal and that their interactions might have driven the diversification in both groups (Ehrlich & Raven 1964). Key interpretations from this theory are that closely related species of plants share similar defenses and support a community of closely related species of insect

herbivores. However, the few studies that have tested these critical assumptions have found contradictory results.

In Chapter 3, we present evidence for enemy-related differentiation among closely related species within two clades in the genus of trees *Inga.* We hypothesize that herbivores may exert divergent selection on defenses, such that closely related plant species will be more different in defensive than in non-defensive traits. Contrary to much coevolutionary theory, we found that sister *Inga* species are more divergent in anti-herbivore defenses than in non-defense traits (e.g., for habitat use and resource acquisition). Furthermore, the assemblages of insect herbivore communities are dissimilar between the populations of coexisting *Inga* species. Taken together, our results suggest that herbivores may have played a significant role in selecting for their phenotypic divergence and potentially in their diversification and coexistence.

In Chapter 4 we provide a more rigorous scrutiny of the coevolutionary hypothesis proposed by Ehrlich & Raven which has dominated our understanding of plant-insect interactions during the last 50 years. A particularly significant contribution of my research is the inclusion of extensive anti-herbivore traits. These are ignored in most other studies and, in their absence, alternative hypotheses cannot be rigorously compared. Specifically, for 38 *Inga* occurring at our study site, we analyze if closely related species of *Inga* are similar in defenses and determine if the herbivore community structure on *Inga* is based on host plant relatedness or on host defensive traits. We also compare the topologies of the phylogenies for both groups. Our results show that close relatives in *Inga* are highly dissimilar in defenses, that shared host plant defensive traits are more important than phylogenetic association in the community structure of the herbivores, and

that host use is conserved. But host use is not conserved in the classical sense, because closely related herbivores feed on plants that are similar in defensive traits but are not necessarily closely related. In other words, the topologies of their phylogenies are not congruent, suggesting that divergence in the traits of herbivore species might not be driven by their interactions with their *Inga* host plants and that closely related species of herbivores may diverge slowly in the traits that determine host choice.

When taken together, these results show that, as expected, plant defenses determine host choice. But, they also strongly suggest that plant-antiherbivore defenses evolve rapidly and that herbivore traits involved in host choice evolve more slowly and depend more on existing host-choice traits. Hence, there is an apparent asymmetry in the interaction between *Inga* and its herbivores. Specifically, although divergence in herbivores might not be driven by their interactions with plants, herbivores may be an important factor driving the divergence among plant species.

Significance of the study

Results from my project provide mechanistic explanations about how plant defenses determine phytophagous insect host associations. They also shed light on the process of evolution, and lead to a reevaluation of the classic expectations from the coevolutionary theory of the arms race. On an ecological scale, my study provides evidence that herbivore pressure may favor neighbors that are dissimilar in their defenses. These insights will address fundamental questions about how so many species can coexist and what processes may have facilitated the evolution of the great diversity of plants and herbivores in tropical rainforests.

In addition to contributions to fundamental questions in ecology and evolution, a better understanding of *Inga* may provide practical applications in agroforestry. Many species of *Inga* have multiple uses on small-scale farms. Their fruits are edible and the wood is used domestically or marketed for fuel in many Latin American countries. More than 30 species are used as shade trees for perennial crops in agroforestry, such as cacao and cafe plantations. Many other benefits from *Inga* have been also reported, such as the fertilizing effect of leaf litter and mulch, nitrogen fixation by their symbiotic rhyzobia, maintenance of soil moisture, erosion control and weed suppression. Therefore, *Inga* is one of the most promising tree genera for agroforestry. By identifying *Inga*-specific herbivores and understanding how *Inga* defends against them, data from my dissertation could help to identify species combinations that differ in their defenses and do not share herbivore species. This may reduce herbivory under agroforestry situations. Additionally, *Inga* provides nectar on the leaves to attract predatory ants that protect the leaves by eating herbivores. My data could identify *Inga* species that are particularly effective at attracting ants. Ants attracted to *Inga* nectar may also reduce herbivory on associated shade crops. Thus my basic research should help inform and improve agroforestry use of this popular tree species.

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CHAPTER 2

THE RESOURCE AVAILABILITY HYPOTHESIS REVISITED: A META-ANALYSIS

Functional Ecology

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EVOLUTIONARY ECOLOGY OF PLANT DEFENCES

The resource availability hypothesis revisited: a meta-analysis

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Summary

1. Several theories have provided a framework for understanding variation in plant defence against herbivores. Among them, the plant apparency theory and the resource availability hypothesis (RAH) have aimed to explain the patterns of defence investment and the selective pressures that have led to the variety of defensive strategies across species. Here we provide a historical review of both theories, present evidence that shaped their development and contrast their predictions.

2. We present the results of a meta-analysis of the utility of the RAH 25 years after it was proposed and compare it to apparency theory. We performed a meta-analysis of 50 studies that have examined plant growth, defences and herbivory in relation to resource availability across latitude and ontogeny. Specifically, we tested four predictions that follow the RAH: (i) species adapted to resource-rich environments have intrinsically faster growth rates than species adapted to resource-poor environments; (ii) fast-growing species have shorter leaf lifetimes than slow-growing species; (iii) fast-growing species have lower amounts of constitutive defences than slow-growing species; and (iv) fast-growing species support higher herbivory rates than slow-growing species.

3. Our results confirm the predictions that species adapted to resource-poor environments grow inherently more slowly, invest more in constitutive defences and support lower herbivory than species from more productive habitats. Our data also showed that variation in growth rate among species better explains the differences in herbivory than variation in apparency, suggesting that the evolution of different defensive strategies across species is resource, rather than herbivore driven. We also found that the application of this theory appears robust across latitude and ontogeny, as the magnitude of the effect sizes for most of the predictions did not vary significantly between ecosystems or across ontogenic stages.

4. We conclude that the RAH has served as a valid framework for investigating the patterns of plant defences and that its applicability is quite general.

Key-words: habitat resources, herbivory, meta-analysis, plant apparency, plant defences, plant defence theory, plant growth, resource availability hypothesis

Introduction

Because plants and herbivores constitute over half of the macroscopic diversity on earth, their interactions play a fundamental role in biodiversity and ecosystem function. For example, the diversity of plant species coexisting at a single site may frequently be shaped by the negative density- and distance-dependent effects of herbivores (Janzen 1970; Connell 1971). Additionally, the evolutionary trajectory of both plant and herbivore traits is driven by the 'arms race' where plants are under continual selection to optimize defence investments and herbivores respond with counter adaptations to detoxify or avoid the defences (Ehrlich & Raven 1964; Thompson 1988). In this paper, we provide some historical context for the development of theories that have aimed to explain the patterns of defence investment and the selective pressures that have led to the variety of defensive strategies across species. We focus on two main

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theories, plant apparency theory (Feeny 1976) and the resource availability hypothesis (Coley, Bryant & Chapin 1985), present evidence that shaped their development and contrast their predictions. We then present results from a meta-analysis of studies examining interspecific variation in defence to assess the utility of these theories.

Although plants were credited with having effective anti-herbivore defences as early as 1888 (Stahl), it was not until Dethier's (1954) and Fraenkel's (1959) papers that the significance of plant secondary metabolites was widely appreciated. Since then, the details of myriad defensive traits and the concept of bottom-up control of herbivores have permeated the literature (Ehrlich & Raven 1964; Whittaker & Feeny 1971; Levin 1976; Haukioja 1980; Lindroth & Batzli 1984; Power 1992). While the concept of plant defences was embraced, the puzzle remained as to why the amount and type of defence differed so much among species. Fundamental to explaining interspecific variation in defences is, understanding the costs and benefits of defensive traits. The costs of defence have been extensively studied, and although they have occasionally been difficult to quantify, many examples of direct, indirect and ecological costs have been documented (Simms 1992; Koricheva 2002; Strauss *et al.* 2002). The benefits of reduced herbivory, while not universal, have also been shown (Marquis 1984; Belsky 1986). Most of the synthetic theories addressing interspecific differences in defence assume that selection has optimized investments, such that the benefits outweigh the costs (Feeny 1976; Grime 1977; McKey 1979; Rhoades 1979; Coley, Bryant & Chapin 1985; Crawley 1985). In the next section we focus on two of these theories, apparency theory and the resource availability hypothesis that sought explanations for why species differed in their investment in defences. We start with a historical review of the theories and the evidence that shaped their development.

Apparency theory

The first major attempt to identify interspecific patterns of plant defences and to infer the processes responsible was apparency theory (Feeny 1976). A similar idea was simultaneously presented by Rhoades & Cates (1976). This theory revolutionized the field, as it shifted the focus from cataloguing the array of defensive traits, to asking *why* species differed in defences. The theory not only identified patterns of defences but suggested that the apparency of species to herbivores was the cause. Feeny posited that species that were long-lived would be apparent or 'bound to be found' by both generalist and specialist herbivores and therefore would be under strong selection for effective defences against both. The high investments in secondary metabolites, such as tannins in oaks, were consistent with this. Tannins were thought to reduce digestibility of leaves by binding with proteins, a mechanism of action that would be difficult for herbivores to circumvent. Feeny referred to these types of defences as 'quantitative' because the greater the investment, the more effective they would be. Furthermore, he posited that they would present an effective defence against all herbivores, both specialists and generalists. In contrast, he suggested that unapparent species were short-lived and ephemeral in time and space, and because of this unpredictability, it would be difficult for herbivores to specialize on them. Thus, unapparent species could evade specialists and would only need defences that were effective against generalists. Using herbaceous crucifers as an example, he called these 'qualitative' defences. Qualitative defences of apparent plants were typically present in low concentrations and were low molecular weight molecules such as sinigrins and alkaloids. They were thought to act on specific animal targets and present significant barriers to generalists and non-adapted insects. Although Feeny hypothesized that it would be possible for herbivores to evolve counter adaptations to qualitative defences, the opportunity for specialization would not arise because unapparent plants were ephemeral and unreliable food sources. Unapparent plants would therefore escape from specialists and have qualitative defences against generalists. Apparent plants would have quantitative defences that would be effective against both generalists and specialists. Thus, the apparency of plants would determine if they were attacked by specialist herbivores or not, and the herbivores in turn would determine which defences, quantitative or qualitative, were optimal. Because of the elegance of apparency theory and the plausible fit with nature, the theory was rapidly accepted and profoundly shaped the field. It has been cited 1400 times and established the paradigm against which subsequent theoretical and empirical work has been judged.

Resource availability hypothesis

The resource availability hypothesis (RAH), also called the growth rate hypothesis (Coley 1987; Stamp 2003), accepted Feeny's premise that long-lived species (apparent) invested heavily in defences and short-lived species (unapparent) did not, but presented an alternative explanation of the mechanism. Coley, Bryant & Chapin (1985) proposed that the observed range of defence investment was not due to differences among species in apparency, but to differences among species in the cost/benefit ratio of defences. They argued that the costs and benefits of investing in defence depended on the inherent growth rate of the species. In a fast-growing species, the opportunity cost of investing in defence would be high, as reallocating resources from photosynthetic leaves would have a much bigger negative impact on a fast grower compared to a slow grower. However, for fast growers, the negative impact of losing leaf area would be low, as they could more quickly replace lost leaves and a given amount of damage would represent a smaller percentage of their annual growth. Furthermore, the RAH postulated that herbivore pressure was a characteristic of the environment, rather than of a species' apparency, and that even if the *risk* of herbivory were uniform across species, selection could favour different levels of defence in species with different inherent growth rates. This is because the inherent growth rate determines the opportunity cost of defence and the impact of herbivory.

The quantitative expression of these thoughts led to the model: $dC/dt = G \times C \times (1-kD^{\alpha})-(H-mD^{\beta})$. Here, the realized growth of a plant (dC/dt) depends on the inherent growth rate G (g $g^{-1}day^{-1}$) times the plant biomass at time zero (C g) minus investments in defence ($1-kD^{\alpha}$) and losses to herbivores ($H-mD^{\beta}$). The inherent growth rate (G) of a species is determined by the resource levels of the habitat to which it is adapted, regardless of herbivore pressure. For example, species from nutrient-poor or low-light habitats grow slowly, even if moved to better conditions because the best adaptation to a low-resource environment is to have a low demand for resources (Grime 1977; Chapin 1980). D (g g^{-1}) is the defence investment expressed as a proportion of plant biomass, because this reflects allocation by the plant and impact on herbivores. The growth reduction due to defences ($1-kD^{\alpha}$) reflects the amount of investment and the opportunity costs, where k (g day^{-1}) and α are constants that relate an investment in defence (D) to a reduction in growth. The rate of herbivory H (g day^{-1}) drops as defences increase (mD^{β}), where m (g day^{-1}) and β are constants that determine the shape of the defence–effectiveness curve. In the simplest model, the maximum possible rate of herbivory (H) is held constant, although this can be changed to reflect differences across habitats. However, even holding maximum herbivory constant, and by changing only the inherent growth rate, we shift the level of defence that maximizes the realized growth (Fig. 1). Thus, for fast-growing species the optimal defence level is low, even though this leads to high rates of herbivory. For slow-growing species, the optimal defence level is high, even though this cost further reduces the realized growth rate.

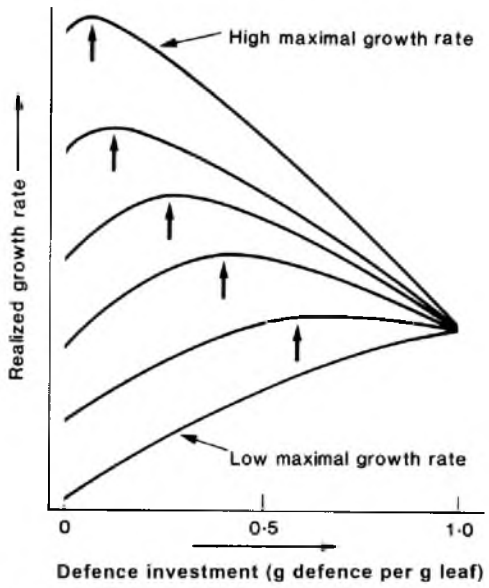

Fig. 1. Effects of defence investment on realized growth. Each curve represents a plant species with a different maximal inherent growth rate. Levels of defence that maximize growth are indicated by arrows. From Coley, Bryant & Chapin (1985).

The RAH was inspired by independent research in rain forests in Panama and boreal forests in Alaska. Coley (1983) undertook to test apparency theory in the tropical rain forest, where the high species diversity allowed replicates of 41 species. She classified gap-requiring trees as unapparent since they were short-lived and only occurred in light gaps. Shade-tolerant trees were spread throughout the forest in both gaps and understorey, were long-lived and were considered apparent. As predicted by apparency theory, the pioneers invested less in defences, however, they did not escape damage, but on average had six times the rates of herbivory as compared to shade-tolerant species even though all plants were measured in the same gap habitats. Furthermore, there was no difference in the variance of herbivory for gap and shade species, although the variance should have been higher for gap species if some individuals were escaping discovery by specialist herbivores and others were found and heavily eaten. Thus apparency did not seem to explain the differences in defence strategies. Instead, there was a strong correlation between investment in defences and the amount of herbivory ($r^2 = -0{\cdot}52$, $P < 0{\cdot}001$) confirming that defences did reduce herbivory and that plants benefited in proportion to their investment. There was also a strong negative relationship between the growth rate of a species and the investment in defences ($r^2 = -0{\cdot}69$, $P < 0{\cdot}01$) suggesting that differences in growth rates might be important determinants of defence costs and benefits. These results paralleled those found by Bryant and Chapin in the boreal forest with hares browsing on dormant twigs (Bryant & Kuropat 1980; Bryant, Chapin & Klein 1983). Again apparency did not seem to differ among species but there were strong correlations between plant growth rate and both defence investment and herbivory. The strikingly similar patterns observed in these two very different ecosystems inspired the collaboration that led to the RAH.

Other theories

In this paper we focus on theories aimed at explaining differences *among* species in defences. Both apparency theory and the RAH have proposed possible reasons selection may have led to a range of optimal defences *across* species. Elements of the RAH have also been developed previously by Janzen (1974), Grime (1977, 2001) and Mattson (1980). Alternative approaches suggest that interspecific differences could arise due to variation in the extent of sexual reproduction (Levin 1975; Johnson, Smith & Rausher 2009) or to selection for escalation during radiations in tightly co-evolved systems (Farrell, Dussourd & Mitter 1991; Agrawal & Fishbein 2008). In addition, there are other influential theories that have been effective in helping us to understand defence differences among individuals *within* a species. Although these differences can arise through divergent selection, they are more frequently the result of plastic responses within a species to environmental gradients. Most notable are the carbon-nutrient balance hypothesis (Bryant, Chapin & Klein 1983) and the growth-differentiation balance hypothesis (Herms & Mattson 1992). Plants can also respond to herbivory by

inducing production of defences (Karban & Myers 1989; Karban 2011). Another strategy is to tolerate herbivore damage by storing sufficient resources to allow regrowth (Strauss & Agrawal 1999). We do not discuss these ideas, as the goal of this paper is to review theories whose main objective was to understand interspecific differences in constitutive defences.

However, it is worth noting that there is much confusion in the literature regarding the predictions of some of the above mentioned theories and the circumstances under which they are applicable (Stamp 2003). In our literature review, it was common to find studies claiming they supported the RAH when they did not, and others refuting the theory when their results were in agreement. The RAH was also frequently invoked when comparing phenotypic responses of plants to different environments, even though the RAH explicitly refers to optimal levels of defence that have evolved in species adapted to different environments. Although phenotypic plasticity theoretically could mirror adaptations seen across species, they often do not, but instead seem to reflect imbalances in allocation. The carbon-nutrient balance hypothesis (CNB), which was designed to explain these phenotypic shifts in defences, does not assume optimality and therefore makes different predictions than the RAH (Bryant, Chapin & Klein 1983). Conversely, the growth-differentiation balance hypothesis (GDBH), as elaborated by Herms & Mattson (1992), does assume that phenotypic variation in secondary metabolism represents adaptive plasticity consistent with predictions of optimal defence theory (see also Glynn *et al.* 2007). Furthermore, we found that the CNB hypothesis was often misused to explain interspecific differences, as did Stamp (2003) for GDBH. Bryant, Chapin & Klein (1983) and Herms & Mattson (1992) addressed both phenotypic and evolutionary responses of plants to resource availability, which no doubt has contributed to this confusion.

Contrasting plant apparency and RAH

Because the theories of resource availability and apparency are not mutually exclusive and in some cases make similar predictions, in the next section of the paper we examine the generality and utility of the RAH 25 years after it was proposed and, compare it to apparency theory. We examined interspecific patterns of growth, defence and herbivory by means of meta-analyses based on 50 studies published between 1985 and 2010 and conducted on >600 different plant species (see references of studies included in Appendix S1, Supporting information). Specifically, we performed separate meta-analyses for each of the four predictions from the RAH: (i) species adapted to resource-rich environments have intrinsically faster growth rates than species adapted to resource-poor environments; (ii) fast-growing species have shorter leaf lifetimes than slow-growing species; (iii) fast-growing species have lower amounts of constitutive defences than slow-growing species; and (iv) fast-growing species support higher herbivory rates than slow-growing species. We examine these predictions across latitude and ontogeny. We selected for relevant studies using Web of Knowledge, Google Scholar and Web of Science, searching for the terms 'plant' and 'herbiv*' and 'defens*' and 'resource*' (or 'light' or 'nutrient*') and 'growth'. Other relevant studies were found by searching the reference section in the articles retrieved from the term searches. We restricted our analyses to studies that examined *interspecific* differences in plant species within a site or between sites differing in their degree of resource availability. Thus, studies that compared growth, defences or herbivory in the same plant species in different resource environments were not considered. For a complete description of our inclusion criteria see methods in Appendix S2 (Supporting information). For the last two meta-analyses (predictions 3 and 4 from the RAH), the articles were grouped into two types: studies that compared investment in plant defences, herbivory and growth between two or more *different* species within a site, and those that compared two or more *different* species growing in sites with divergent resource levels (light and nutrients, see Appendix S3, Supporting information for further categorization of studies included in these meta-analyses). In the original articles, the habitats in which the studies were conducted were usually classified as either resource-poor environments or resource-rich environments based on the levels of nutrient availability or of light availability. All the meta-analyses were conducted with the program MetaWin version 2.1.5 (Rosenberg, Adams & Gurevitch 2000), and using the mixed effects model (Gurevitch & Hedges 1993; see Appendix S2, Supporting information for a complete description of materials and methods and Appendix S3, Supporting information for effect sizes).

Results

The results that follow are based primarily on studies of woody terrestrial species. Although we did not specifically exclude studies of herbaceous plants, or of marine and aquatic plants, most of these studies evaluated intraspecific differences in growth, defences and herbivory, and as such, did not meet our inclusion criteria (see Appendix S2, Supporting information).

PREDICTION 1: SPECIES ADAPTED TO RESOURCE-RICH ENVIRONMENTS HAVE INTRINSICALLY FASTER GROWTH RATES THAN SPECIES ADAPTED TO RESOURCE-POOR ENVIRONMENTS

Overall, we found that species from resource-rich environments grew faster than those from resource-poor environments (d = 2·75, 95% CI = 1·01–4·85, n = 24, n_{fs} = 232; Fig. 2; Table 1 in Appendix S3, Supporting information). We did not find significant variation among studies conducted in tropical forests vs. temperate forests (Q_B = 0·6, d.f. = 1, P = 0·59), nor among ontogenic stages (Q_B = 7·07, d.f. = 2, P = 0·33). However, we found that the magnitude of the effect was significantly different among the different growth traits (Q_B = 16·35, P = 0·04). The lower variance was found among those studies that reported growth rate and height. When only these studies were analysed, the results

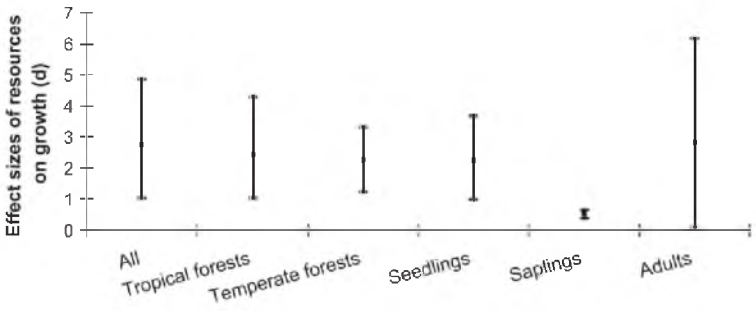

Fig. 2. Mean and 95% confidence intervals for the effect sizes of resources on plant growth measures (weighted standardized mean, Hedges' d): for all studies (n = 24) and for studies conducted only in tropical forests (n = 17), in temperate forests (n = 6), with seedlings (n = 9), with saplings (n = 5), and with adults (n = 8).

were similar to those obtained from the whole data set (d = 1·22, 95% CI = 0·17–2·14, n = 17, n_{fs} = 36·6).

PREDICTION 2: FAST-GROWING SPECIES HAVE SHORTER LEAF LIFETIMES THAN SLOW-GROWING SPECIES

We found a strong and negative effect of growth rate on leaf lifetime (z = −1·78, 95% CI = −2·55 to −1·06, n = 10, n_{fs} = 110; Fig. 3; Table 2 in Appendix S3, Supporting information), confirming the prediction that slow-growing species have longer leaf lifetimes than fast-growing species. We were unable to compare the magnitude of the effect between habitats with different resources, different ecosystems or ontogenic stages because most studies included in our meta-analysis were conducted with species in the same site, in tropical forests and with adult individuals (Appendix S2, Supporting information).

PREDICTION 3: FAST-GROWING SPECIES HAVE LOWER INVESTMENTS IN CONSTITUTIVE DEFENCES THAN SLOW-GROWING SPECIES

To test this prediction we conducted two analyses. We analysed growth effects on defences for fast- and slow-growing species in the same habitat to control, at least in part, for differences in the expression of defences across species caused by varied environments. We also compared growth effects between two or more different species in different habitats, which would include plasticity as well as evolved differences for constitutive defences (see methods in Appendix S2, Supporting information). The studies only reported quantitative results for phenolic compounds and terpenes, but not for other classes of chemical defences (see Table 3 in Appendix S3, Supporting information).

Effect of plant growth on investment in plant defences between species within a site

As predicted by the RAH, when all types of defences were considered together (chemical and mechanical), fast-growing species invested less in constitutive defences than slow-growing species (z = −0·52, 95% CI = −0·66 to −0·38, n = 57, n_{fs} = 1824·6; Fig. 3). This effect was more pronounced for seedlings and saplings than for adults as differences between seedlings and saplings (−0·61 vs. −0·59) were virtually non-existent (−0·18; Q_B = 9·36, d.f. = 2, P = 0·04), and also for studies conducted in tropical (−0·62) vs. temperate (−0·3) forests (Q_B = 6·44, d.f. = 2, P = 0·04). The result was also significant when considering only chemical defences (z = −0·3, 95% CI = −0·45 to −0·12, n = 23, n_{fs} = 139·6; Fig. 3). The same pattern was maintained for the effect of growth on investment in terpenes (z = −0·43, 95% CI = −0·7 to −0·23, n = 4, n_{fs} = 105·7; Fig. 3) and total phenolics and tannins, although it was not significant for the last two. We also found that fast-growing species invested less in mechanical defences (z = −0·85, 95% CI = −1·2 to −0·59, n = 25, n_{fs} = 584·2; Fig. 3), and the magnitude of the effect was significantly higher in seedlings (−1·67) vs. saplings (−0·64) and adults (−0·33; Q_B = 26·29, d.f. = 2, P = 0·004). Our meta-analysis also confirmed that fast-growing species had higher leaf

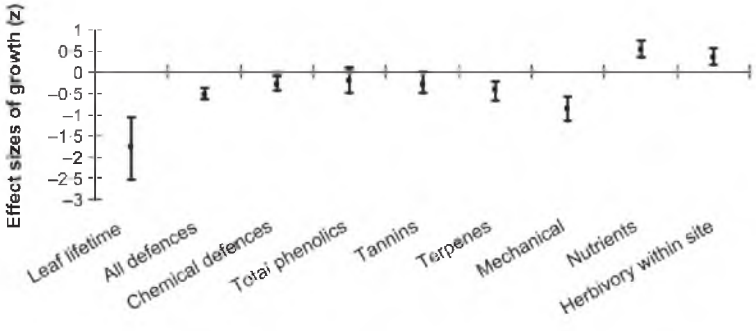

Fig. 3. Mean effect sizes (z) and 95% confidence intervals for growth rate effects on investment in plant defences and herbivory between species within a site. The dependent variables include: leaf lifetime (n = 10), all constitutive defences (n = 57), all chemical defences (n = 23), total phenolics (n = 6), tannins (hydrolysable and condensed; n = 12), terpenes (n = 4 records from one study by Fine, Mesones & Coley 2004), mechanical defences (n = 6), nutrient content (n = 25) and herbivory (n = 16).

nutrient content (z = 0·51, 95% CI = 0·33–0·72, n = 6, n_{fs} = 42·5; Fig. 3).

Effect of plant growth on investment in defences between species in habitats with different nutrient availability

Our meta-analyses showed that studies comparing investment in defences between two or more different species from habitats with different levels of nutrients had contradictory results compared to within site comparisons. In these studies, there were no differences between fast-growing and slow-growing species in defence investment when all defences were combined (chemical and mechanical). We found the same result when all chemical defences were combined into one response variable (total phenolics, hydrolysable tannins and condensed tannins), or individually for tannins and leaf toughness (Fig. 4). There were not enough studies to compare trichomes. However, fast-growing species invested more in total phenolics (z = 0·85, 95% CI = 0·04–1·41, n = 8, n_{fs} = 0; Fig. 4). We did not find differences in the effect of growth on defences between studies conducted in tropical vs. temperate forests, nor among studies performed with seedlings or saplings or adult individuals. Since only one of our selected studies compared nutrient content in leaves between species from habitats with different nutrient levels we did not conduct a meta-analysis for this trait (Appendix S1, Supporting information).

Effect of plant growth on investment in defences between species in habitats with different light availability

Although in general slow-growing species invested more in defences than fast-growing species, this difference was not significant when comparing different species from habitats with different levels of light (95% CI = −0·35 to 0·64; Fig. 5). Individually, we found a significant negative effect of growth on mechanical defences (only leaf toughness, as there were not enough studies comparing production of trichomes) (z = −0·82, 95% CI = −1·42 to −0·13, n = 8, n_{fs} = 18·8) but not on other defences. There were no differences in studies conducted in different ecosystems and with different ontogenetic stages.

PREDICTION 4: FAST-GROWING SPECIES SUPPORT HIGHER HERBIVORY RATES THAN SLOW-GROWING SPECIES

When comparing different species within the same site, we found that fast-growing species suffered higher herbivory compared to slow-growing species (z = 0·35, 95% CI = 0·15–0·55, n = 16, n_{fs} = 27·4; Fig. 3; Table 4 in Appendix S3, Supporting information). In contrast, the comparison across sites includes not only differences among species in their growth rate, but also differences among sites in overall herbivore pressure. In this comparison, herbivory for fast growers in resource-rich sites was higher than for slow growers at resource-poor sites, but the effect was not significantly different from zero (z = 0·29, 95% CI = −0·18 to 0·68, n = 29, n_{fs} = 0).

Discussion

The goal of both apparency theory and the RAH has been to provide a theoretical framework that adequately explains the interspecific variation in plant defensive strategies. The RAH relates the evolution of defences to interspecific differences in

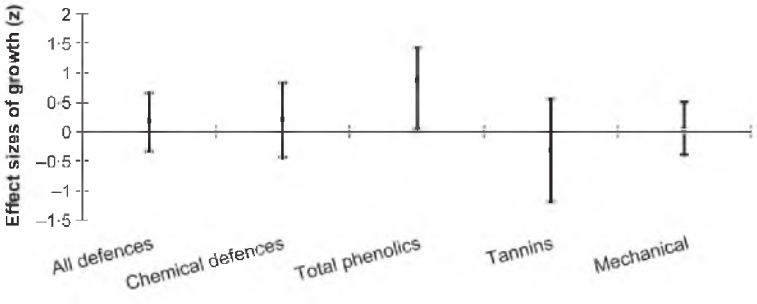

Fig. 4. Mean effect sizes (z) and 95% confidence intervals for growth rate effects on investment in plant defences between species in habitats with different nutrient availability. Dependent variables include: all defences (n = 24), chemical defences (n = 18), total phenolics (n = 8), tannins (n = 10) and mechanical defences (n = 6).

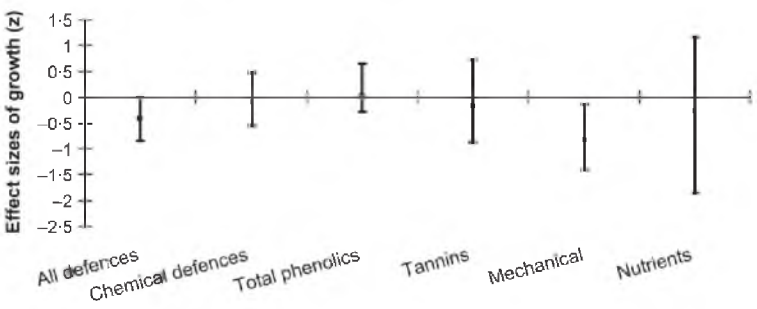

Fig. 5. Mean effect sizes (z) and 95% confidence intervals for growth rate effects on investment in plant defences between species in habitats with different light availability. Dependent variables include: all defences (n = 17), chemical defences (n = 9), total phenolics (n = 4), tannins (n = 5), mechanical defences (n = 8) and nutrient content (n = 2).

inherent growth rate, whereas apparency theory assumes that defences are related to a species' predictability to herbivores. Although the theories have different assumptions regarding the reasons leading to defence differences, some of the predictions are similar. For example, both theories agree that long-lived, slow-growing species (apparent species) should invest more in defences than short-lived, fast-growing species (unapparent species). However, a fundamental difference between the theories is their contrasting predictions for the amount of herbivory. The RAH predicts that fast-growing species should suffer greater herbivore damage, while apparency theory predicts similar losses for apparent and unapparent species. In the discussion that follows, we examine results for defence and herbivory, as these apply to both theories. We also examine two predictions that apply only to the RAH, that resources affect growth and that growth affects leaf lifetimes.

PREDICTION 1: RESOURCE EFFECTS ON PLANT GROWTH RATE

Our meta-analysis suggests that, in agreement with the RAH, plant species from resource-rich environments had higher growth rates than species from resource-poor environments (Fig. 2). These patterns hold across different ecosystems and ontogenic stages, as we did not find significant differences between studies conducted in tropical forests vs. temperate forests and in seedlings, saplings and adults. It is less certain whether these patterns will also hold for herbaceous species since all the studies included in our meta-analysis were based on woody species. However, a similar association between resources and inherent growth was found in a meta-analytical study performed with temperate herbs (Taub 2007). Our results are consistent with the well-established fact that species growth rates vary with fertility levels (Grime 2001) and light requirements (Swaine & Whitmore 1988). High rates of growth are hallmark characteristics of plant species adapted to high-resource environments (Grime 1979; Chapin 1980; Lambers & Poorter 1992). In contrast, species adapted to low-resource environments grow slowly and retain their growth habit even under high-resource conditions (Grime 2001).

PREDICTION 2: GROWTH RATE EFFECTS ON LEAF LIFETIME

As predicted, slow-growing species have leaves with significantly longer leaf lifetimes than fast-growing species. Long-lived leaves minimize nutrient losses (Aerts 1995) and constitute an essential adaptation of slow-growing species to habitats with low-resource availability (Grime 1977). The relationship between growth rate and leaf life span was the foundation for suggesting that qualitative defences, because of a higher maintenance cost, would be favoured in leaves with short life spans, and quantitative defences, with high initial costs but low maintenance costs, would be favoured in leaves with long life spans (Coley 1987).

PREDICTION 3: GROWTH RATE EFFECTS ON DEFENCES

Both theories predicted greater investment in defence for slow-growing species, but for different reasons. The RAH predicts that for slow-growing species the opportunity cost of defence will be low and the negative impact of herbivory high. Therefore, slow growers should exhibit higher investments in constitutive defences (Coley 1987). Apparency theory predicted that apparent plants would need effective defences against both specialists and generalists. The results from our meta-analysis found that, when considering only the studies that compared defence investment across species in the same habitat, there was a significant negative effect of growth rate on overall defence investment. This result was also maintained when considering chemical and mechanical defences independently (Fig. 3). Moreover, this pattern appears robust, as the direction of the growth effect on defences was the same when comparing different latitudes and ontogenetic stages.

Although defences were universally higher in slow growers, our meta-analysis showed that defence differences between fast and slow growers were significantly greater in tropical ecosystems. Possible explanations for this pattern might lie in the fact that, in the tropics, there is a higher absolute investment in defences (Coley & Aide 1991), a higher variance in defensive compounds (Gauld & Gaston 1994), and a greater range of plant growth rates (Van Zandt 2007). Greater amounts and ranges could facilitate detection of differences. Similarly, there was a negative effect of growth on overall defences for all ontogenetic stages, but the magnitude of this effect was significantly higher for seedlings. The reason for this is unclear, however, again, it may be easier to detect differences in defences if seedlings invest more than other age classes because of the potentially devastating effects of herbivory (Barton & Koricheva 2010; but see Boege & Marquis 2005).

However, when analysing the studies comparing two or more different species from different sites, we did not find a significant effect of growth rate on overall defences. This was consistent whether habitats differed with respect to nutrients or light. We interpret this as resulting from a combination of phenotypic responses of plants to short-term changes in resources with selection for different defence strategies in different habitats. Thus, these results can be better explained by integrating both the RAH and the carbon-nutrient balance hypothesis (CNB; Bryant, Chapin & Klein 1983; Dyer & Coley 2002; Stamp 2003). The CNB hypothesis suggests that when resources are in excess of what can be used for growth, they will be invested in defences. Accordingly, under high light where carbon is in excess relative to nutrients, this theory predicts higher amounts of carbon-based defences, whereas the RAH predicts lower defences for species adapted to this low-resource condition. Because of these counterbalancing influences, we would expect no significant effect of plant growth on defences, and this is what we found in our meta-analysis for studies comparing species from sites with different levels of light. In another study, Baldwin & Schultz (1988)

also found no significant differences in phenol investment when comparing species of the genus *Piper* from gaps and understorey. For mechanical defences, the CNB theory does not have a prediction, while the RAH predicts lower mechanical defences for species adapted to high-light levels. Again, our results were consistent with this, as leaves of slow-growing species were significantly tougher.

In contrast to the defence comparisons across light gradients, which were consistent with the combined effects of RAH and CNB theories, results from habitats with different nutrient levels were confusing. Under high nutrient levels, both the CNB hypothesis and the RAH predict lower carbon-based defences, however, we found a non-significant opposite trend. Another meta-analysis (Koricheva 1998) also found a weak but negative effect of fertilization on carbon-based defences. Although Herms & Mattson (1992) proposed a model that integrates genetic and phenotypic plasticity, the predictions are nonlinear and complex, making it difficult or impossible to capture secondary metabolic responses to variation in resource availability (Stamp 2003). Thus, when comparisons are made within a site, there is a clear negative relationship between plant growth and defence following the RAH, however, when confounding effects of environmental plasticity are included (Figs 4 and 5), particularly those associated with nutrient gradients, it is obvious that our understanding is incomplete.

The RAH also predicts higher inducible defences in fast-growing species. This is because the opportunity cost of defence is higher for fast growers, and because fast growers may more often occur under conditions that favour induction, such as predictable, but periodic herbivore attack (Karban 2010). Although we did not analyse this prediction, supporting evidence has been found. In a literature review of 68 studies, Nykanen & Koricheva (2004) found that the production of phenolics and protein-precipitation capacity of tannins increased in fast-growing species after herbivore damage more than in slow-growing species. Van Zandt (2007) found a similar result in an experimental study with nine species of temperate herbaceous plants.

PLANT DEFENCES: UNANSWERED QUESTIONS

A pattern first identified by Feeny was that unapparent plants invested in qualitative defences and apparent plants in quantitative defences. Although this observation has been fairly well supported, the reasons why are still unclear. A quantitative review of defensive classes in different plant guilds corroborated this idea by finding that fast-growing plants (apparent plants) are most often defended with quantitative, dose-dependent defences and slow-growing plants (unapparent plants) with qualitative defences (T. Massad & L. Dyer, pers. comm.). Feeny suggested it was because quantitative defences worked against all herbivores, while qualitative defences worked only against generalists and non-adapted specialists. However, the fact that the herbivores attacking apparent and unapparent plants are a similar mix of specialists and generalists (Futuyma & Gould 1979), and that quantitative and qualitative defences do not have different effects on generalists vs. specialists herbivores (Smilanich 2008; Carmona, Lajeunesse & Johnson 2011; T. Massad & L. Dyer, pers. comm.) brings this explanation into question. Another criticism of apparency theory, though one that we do not view as a fatal attack, is that the primary function of tannins may not be to bind proteins and reduce digestion (Bernays 1981). Instead, more recent evidence shows that oxidation of hydrolysable tannins forms reactive oxygen species, which can overwhelm the antioxidant defences of herbivorous insects and damage midgut tissues (Martin, Martin & Bernays 1987; Appel 1993; Summers & Felton 1994; J. Salminen & M. Karonen, unpublished). Nonetheless, this could be considered a quantitative defence as higher concentrations of hydrolysable tannins will lead to greater levels of oxidative stress.

The RAH proposed that leaf lifetime, which is related to plant growth rates, is the key factor directing selection for the type of defence. They argued that qualitative defences, in addition to being present in low concentrations, are low molecular weight molecules with high turnover or maintenance rates. In contrast, quantitative defences such as condensed tannins, would require a considerable initial investment since they are present at high concentrations, but because they do not turnover, there would be no subsequent maintenance costs. Thus, for species with short-leaf lifetimes, it would be more cost effective to invest in qualitative compounds, whereas for long-lived leaves, the cumulative cost would be lower for quantitative compounds. However, this argument rests on differences in turnover rates for qualitative and quantitative compounds, an assumption that also has been challenged (Mihaliak, Gershenzon & Croteau 1991; Baldwin & Ohnmeiss 1994; van Dam *et al.* 1995; Salminen & Karonen 2011). Thus, the underlying factors favouring compounds along the quantitative/qualitative continuum remain to be determined.

PREDICTION 4: GROWTH RATE EFFECTS ON HERBIVORY

One of the key differences between the RAH and apparency theory is related to the predicted herbivore damage. Apparency theory (Feeny 1976; Rhoades & Cates 1976) predicts similar rates of damage. Unapparent plants escape from specialists and have secondary metabolites that are effective against generalists, whereas, apparent plants have metabolites that are effective against both specialists and generalists. In contrast, RAH predicts that fast-growing species will support higher levels of herbivory than slow-growing species because they are less defended. Our results support the last prediction, since we found a negative and significant effect size of growth rate on herbivory when analysing studies comparing species with different growth rates within the same habitat. Thus, unapparent plant species (fast-growing species according to the RAH) did not escape from herbivory, but had significantly higher levels than apparent species (slow-growing species according to the RAH). Therefore, variation in growth rate among species explains better the differences in

herbivory than variation in apparency. We found similar trends in the meta-analyses for studies comparing herbivory and growth rate between species growing in sites with different level of resources. This comparison not only takes into account differences in growth rates, but also differences between sites in overall herbivore pressure. A negative effect size of growth suggested that fast-growing species from resource-rich habitats suffered higher herbivory than slow-growing species from resource-poor habitats. However, the greater variance and absence of significance is consistent with herbivore pressure varying among habitats.

In addition to high herbivory on unapparent, fast-growing species, there is no evidence that they are attacked more by specialists than ephemeral species (Futuyma & Gould 1979; Cates 1980; Basset 1992), a key element of apparency theory posited to drive selection for different defence strategies. The host-finding abilities of insect herbivores are sufficiently good that escape from discovery does not appear to occur, except perhaps for extremely ephemeral species or tissues. Thus the patterns of defence first described by Feeny may not be adequately explained by a plant's apparency, as this does not lead to differential attack by specialist vs. generalist herbivores.

Conclusions

Both apparency theory and the RAH have provided testable hypotheses for investigating interspecific variation in patterns of plant defences and have stimulated a multitude of studies. Both have been extremely influential and are widely cited (1400 and 1600 citations respectively). Our evaluation of the generality of the RAH 25 years after its first publication shows strong support for the basic tenets linking resources, plant growth, defence and herbivory. It has been suggested that the predictive power of the RAH is mostly supported in tropical forests, with mixed support in temperate forests (Van Zandt 2007). Although we found a higher mean effect size for all our predictions in the tropics, this difference was significant for only one of the predictions. Therefore, we suggest that the applicability of the RAH is general. In addition, because of its simplicity and wide application, the RAH has provided a coherent framework for the generation of new ideas about plant – insect interactions. For example, it has been proposed that resource availability and enemy release may interact in plant invasions (Blumenthal 2006).

More recent approaches in understanding the origin and maintenance of plant defences are often framed in an explicit phylogenetic context. Other approaches of promise ask mechanistic questions regarding the macroevolutionary trends in plant defences, and how selection by herbivores could influence both the speed and direction of selection. Furthermore, how could these interactions be shaped across species ranges and depend on the mosaic of other interacting species? And finally, can plant – herbivore interactions promote plant diversity by promoting rates of speciation or slowing extinction? New phylogenetic and molecular techniques as well as new theoretical approaches in studying plant – herbivore interactions should further enhance our understanding of these fundamentally important interactions across evolutionary and ecological time-scales.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Studies included in the meta-analyses.

Appendix S2. Materials and methods.

Appendix S3. Meta-analysis records.

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APPENDIX S1: *Studies included in the meta-analyses*

Agrawal, A. A. & Fishbein, M. (2008). Phylogenetic escalation and decline of plant defense strategies. *Proceedings of the National Academy of Science of the United States of America* **105**: 10057–10060.

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APPENDIX S2: *Materials and Methods*

We reviewed published studies comparing growth, defenses and herbivory among different plant species and under different resource environments. We selected suitable studies using three electronic databases: Web of Knowledge and Google Scholar for 1985-2010 and Web of Science for 1997-2009. We searched for the terms "plant" and "herbiv*" and "defens*" and "resource*" (or "light" or "nutrient*") and "growth". Other relevant studies were also found by searching the reference section in the articles retrieved from the term searches. These searches led to a large number of articles (~600) that were examined but only a subset met our criteria (described below) and were, therefore, included in our meta-analyses. Thus our analysis is based on 50 studies published between 1985-2010 and conducted on > 600 different plant species (see references of studies included in Appendix 1).

We set a specific inclusion criterion to choose the studies for our meta-analyses. We restricted our analyses to studies that examined interspecific differences in plant species within a site or between sites differing in their degree of resource availability. Thus, studies that compared growth, defenses or herbivory in the same plant species in different resource environments were not considered. In order to be included in our review, a study had to provide data for growth estimates or growth categorization of the plant species and information related to the resource availability of the habitat at which the study was conducted.

In the original articles, the habitats at which the studies were conducted were usually classified as either resource-poor environments or resource-rich environments based on the levels of nutrient availability (low or high, infertile or fertile) or of light availability (shade or gap, understory or canopy, primary forest or secondary forest). Plant growth estimates included growth measurements (biomass, height, leaf area and leaf production) and growth categorizations (slow growing or fast growing as classified by the authors). Leaf lifetime measurements were expressed as the number of days or months a leaf was monitored until senescence. Antiherbivore defenses included plant traits proven to decrease herbivore consumption and/or herbivore growth and survival. Defenses were classified as chemical (concentrations or percentages of secondary compounds per unit weight of tissue), mechanical (trichome density and leaf toughness) or nutritional (concentrations or percentages of water and/or nitrogen per unit weight of tissue). Herbivory measurements were usually estimated as the percentage of leaf area eaten or biomass consumed.

We performed separate meta-analyses for each of the four predictions from the RAH. For the prediction (1) meta-analysis we used 24 records from 13 studies that compared inherent growth measurements between species from resource-poor environments vs. species from resource-rich environments. For meta-analysis of the prediction (2) we included 10 records from 4 studies that examined leaf lifespan in relation to growth rate. For prediction (3) meta-analysis we used 103 records from 30 studies that contrasted investment of constitutive defenses between fast-growing and slow-growing species. And, for the meta-analysis of the prediction (4), we summarized

the results for 42 records from 25 studies that related herbivore damage with plant growth rate. For the last two meta-analyses, the articles were grouped into two types: studies that compared investment in plant defenses, herbivory and growth between two or more different species within a site, and those that compared two or more different species growing in sites with different resource levels (light and nutrients). In addition, for all the meta-analyses, we further distinguished between studies that were conducted in different ecosystems (tropical vs. temperate forests) and with different ontogenetic stages (adults, saplings and seedlings). We performed these further categorizations in order to analyze for other sources of variation in the expression of plant growth, defenses and herbivory in relation to resource availability.

From most studies, more than one record suitable for our meta-analysis could be recovered. Some studies reported results from more than one response variable (growth, leaf lifespan, defense and herbivory). In these cases, we created four separate data sets corresponding to the four response variables and the results for each response variable were included in different meta-analyses. In addition, if a study reported data for several plant species, we included each species separately in the meta-analysis to avoid statistical problems related to non-independent comparisons.

STATISTICAL ANALYSES

Studies included in the meta-analyses of the first prediction addressed plant growth differences in relation to resource availability by comparing growth between species from resource-rich environments vs. species from resource-poor environments. The outcomes from these studies were usually in the form of mean and standard

deviations or standard errors. As a first step in our meta-analysis, we converted all these estimates into a common measure of effect size, the standardized mean difference statistic, Hedges' *d* (Gurevitch & Hedges 2001), which is a statistic commonly used in meta-analyses (Hawkes & Sullivan 2001; Gómez-Aparicio *et al.* 2004; Maestre, Valladares & Reynolds 2005). For the reported means and variances of growth measures for species from resource-rich and resource-poor environments we directly calculated *d*:

$$d = \frac{(\bar{X}_R - \bar{X}_P)}{S} J$$

where $\bar{X}_R$ and $\bar{X}_P$ are the means, S is the pooled standard deviation and J is a correction term for small sample sizes (Rosenberg, Adams & Gurevitch 2000). Positive values of Hedges' *d* indicate that plant species in high-resource environments grow faster than those in low-resource environments and vice versa.

Studies used for the meta-analyses of predictions (2), (3) and (4) examined the relationships between continuous variation in leaf lifetime, defenses or herbivory and growth. The outcomes from these studies were usually correlation coefficients or regression equations. However, some studies reported *t*, x^2, and F values from statistical tests comparing such variation in relation to growth. For these studies we selected the Pearson product-moment correlation coefficient (*r)* as the common measure of association between leaf lifetime, defenses or herbivory and growth and calculated effect sizes from these coefficients. The correlation coefficient (*r)* is considered an advantageous effect size statistic because it is easy to interpret (Koricheva 2002,

Koricheva, Nykanen & Gianoli 2004), and principally, because most of the commonly used test statistics can be translated into an *r* value (Rosenberg, Adams & Gurevitch 2000). When the results of the studies were reported in the form of correlation coefficients, they were directly included into the data sets. When the associations were examined as regression analyses, we took the square root from the coefficient of determination (R^2). F-statistics, *t*-statistics and x^2-statistics were transformed into *r* following Rosenberg , Adams & Gurevitch (2000). Individual *r* coefficients were z-transformed and weighted by their respective sample sizes. The sign of *r* reflects the patterns of plant defenses or herbivory in relation to growth. If growth decreases plant investment in defenses, *r* will be negative (and vice versa). If growth increases nutritional content and plant herbivory, *r* will be positive (and vice versa).

All the meta-analyses were conducted with the program MetaWin version 2.1.5 (Rosenberg , Adams & Gurevitch 2000), and using the mixed effects model (Gurevitch & Hedges 1993), which assumes that variation observed among studies is due to sampling error and random variation (Koricheva 2002). As we mentioned above, we conducted separate meta-analyses for each prediction following the RAH. The magnitude of the effect size was considered to be statistically significant when the bias-corrected 95% confidence interval of the z-transformed effect size, generated from 9,999 iterations, did not include zero (Gurevitch & Hedges 1993). In addition, following Rosenberg, Adams & Gurevitch (2000), we analyzed if the size of the effect varied across different measurements of growth rate for the meta-analysis of prediction (1), and between studies that were conducted in tropical vs. temperate forests and between different ontogenic stages (seedlings, saplings and adults) for all meta-analyses. For this, we used the

statistic Q_b, which is a weighted sum of squares that can be tested against an x^2 distribution with n -1 degrees of freedom (Gurevitch & Hedges 2001). Significant values of Q_b imply that there are differences in effect sizes between groups.

In order to test publication bias, e.g., the tendency of journals to publish only studies with significant results, we calculated a fail-safe number (n_{fs}) by means of the weighted method proposed by Rosenthal (1979). A fail-safe number is the number of non-significant or unpublished studies needed to change a significant effect to a non-significant effect in a meta-analysis (Rosenberg, Adams & Gurevitch 2000).
If this number is larger than $5n + 10$, where n is the number of observed studies in the meta-analysis, we can be confident of the robustness of our analyses against publication bias (Rosenberg, Adams & Gurevitch 2000).

Another source of bias in meta-analytical methods constitutes the phylogenetic nonindependence. Given that the goal of meta-analysis is to summarize research from multiple taxa, the absence of phylogenetic independence, resulting from shared phylogenetic history among closely related taxa, violates statistical assumptions of independence (Lajeunesse 2009). Thus, the integration of phylogenetic information into ecological meta-analysis is becoming a new and exciting area. However, because of the novelty of its application, the use of phylogenetic information in quantitative reviews still has several theoretical and practical issues, such as the accessibility to information and difficulty of analysis needed to construct evolutionary relationships (Lajeunesse 2009), especially for unbalanced designs like our study. For these reasons, we decided to not include phylogenetic information in our analyses. Moreover, we worked with a broad diversity of plant species from many different families and from many different

geographic localities. Therefore, we consider that the absence of phylogeny will not threaten our conclusions because, by inspection, there is no a phylogenetic bias in our results.

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APPENDIX S3

Meta-analysis records

Table S3.1. Prediction (1) meta-analysis records for the effect of resources on plant growth. N= sample size.

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Effect size (d)	N
Bryant *et al.* 1989	Tropical	Nutrients	Seedling	Woody	6.21	30
Bryant *et al.* 1989	Tropical	Nutrients	Seedling	Woody	19.54	30
Coley, 1987	Tropical	Light	Saplings	Woody	0.58	47
Coley, 1987	Tropical	Light	Saplings	Woody	0.29	47
Coley, 1987	Tropical	Light	Saplings	Woody	0.43	47
Coley, 1988	Tropical	Light	Saplings	Woody	0.76	41
Coley, 1988	Tropical	Light	Saplings	Woody	0.52	41
Cunningham, *et al.* 1999	Temperate	Nutrients	Adults	Woody	1.68	9
Cunningham, *et al.* 1999	Temperate	Nutrients	Adults	Woody	1.68	9
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	-5.88	30
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	0.32	30
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	-4.28	30
Dudt & Shure, 1994	Temperate	Light, Nutrients	Seedlings	Woody	0.33	240
Fine *et al.* 2004	Tropical	Nutrients	Seedlings	Woody	0.13	880
Fine *et al.* 2006	Tropical	Nutrients	Seedlings	Woody	3.8	880
Fine *et al.* 2006	Tropical	Nutrients	Seedlings	Woody	1.96	880
Folgarait & Davidson 1994	Tropical	Light	Seedlings	Woody	1.52	24

Table S3.1. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Effect size (d)	N
Folgarait & Davidson 1994	Tropical	Light	Seedlings	Woody	1.52	24
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	-4.12	25
Read *et al*. 2006	Tropical		Adults	Woody	0.93	43
Read *et al*. 2006	Tropical		Adults	Woody	12.49	43
Shure & Wilson 1993	Temperate	Light	Adults	Woody	2.14	240
Shure & Wilson 1993	Temperate	Light	Adults	Woody	4.87	240

Table S3.2. Prediction (2) meta-analysis records for the effect of growth on leaf lifespan. N= sample size.

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Effect size (z)	N
Coley 1988	Tropical	Light	Saplings	Woody	-0.57	41
Folgarait & Davidson 1994	Tropical	Light	Seedlings	Woody	-0.48	24
Reicht *et al.* 1992	Temperate		Seedlings	Woody	-0.7	287
Reicht *et al.* 1992	Temperate		Seedlings	Woody	-1.42	287
Reicht *et al.* 1992	Temperate		Seedlings	Woody	-0.9	287
Reicht *et al.* 1992	Temperate		Seedlings	Woody	-1.04	287
Lowman 1992	Tropical & Temperate		Adults	Woody	-4.26	4002
Lowman 1992	Tropical & Temperate		Adults	Woody	-2.48	4002
Lowman 1992	Tropical & Temperate		Adults	Woody	-2.64	4002
Lowman 1992	Tropical & Temperate		Adults	Woody	-3.15	4002

Table S3.3. Prediction (3) meta-analysis records for the effect of growth on defense investment. Comparison= Indicates whether the study was used for 1 (within-site) or 2 (among-site) sub-analyses; N= sample size.

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Defense	Comparison	Effect size (z)	N
Agrawal & Fishbein 2008	Temperate		Seedlings	Herbs	Total defenses	-	0.14	190
Almeida-Cortéz *et al.* 1999	Temperate		Seedlings	Herbs	Total phenolics	2	0.42	140
Almeida-Cortéz *et al.* 1999	Temperate		Seedlings	Herbs	Total defenses	2	0.12	140
Baldwin & Schultz 1988	Tropical	Light	Adult	Woody	Total phenolics	1	0.05	35
Baldwin & Schultz 1988	Tropical	Light	Adult	Woody	Condensed tannin	1	0.0042	35
Baldwin & Schultz 1988	Tropical	Light	Adult	Woody	Cinnamic acid	1	-0.14	35
Baldwin & Schultz 1988	Tropical	Light	Adult	Woody	Hydrolysable tannins	1	1.24	35
Coley 1987	Tropical	Light	Saplings	Woody	Proantho-cyanidins	2	-0.56	47
Coley 1987	Tropical	Light	Saplings	Woody	Condensed tannin	2	-0.43	47
Coley 1987	Tropical	Light	Saplings	Woody	Fiber	2	-0.76	47
Coley 1987	Tropical	Light	Saplings	Woody	Lignin	2	-0.44	47
Coley 1987	Tropical	Light	Saplings	Woody	Toughness	2	-0.76	47
Coley 1987	Tropical	Light	Saplings	Woody	Cellulose	2	-0.78	47
Coley 1988	Tropical	Light	Saplings	Woody	Total defenses	2	-0.85	41

Table S3.3. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Defense	Comparison	Effect size (z)	N
Cunningham *et al.* 1999	Temperate	Nutrients	Adults	Woody	Phenolics	1	0.76	9
Cunningham *et al.*1999	Temperate	Nutrients	Adults	Woody	Tannins	1	0.76	9
Cunningham, *et al.* 1999	Temperate	Nutrients	Adults	Woody	Phenolics	1	0.76	9
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	Tannins	2	1.3	30
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	Trichomes	2	-2.96	30
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	Trichomes	2	-3.03	30
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	Trichomes	2	-2.32	30
Dominy *et al.* 2008	Tropical	Light	Adults	Woody	Toughness	1	-0.86	62
Dominy *et al.* 2008	Tropical	Light	Adults	Woody	Toughness	1	-0.83	67
Dominy *et al.* 2008	Tropical	Light	Adults	Woody	Toughness	1	-0.07	50
Dudt & Shure, 1994	Temperate	Light, Nutrients	Seedlings	Woody	Tannins	2	-0.17	240
Eichhorn, Fagan & Compton 2007	Tropical		Seedlings	Woody	Phenolics	2	-1.29	5
Eichhorn, Fagan & Compton 2007	Tropical		Seedlings	Woody	Toughness	2	-1.29	5
Fine *et. al.* 2006	Tropical	Nutrients	Seedlings	Woody	Total phenolics	2	-0.4	294

Table S3.3. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Defense	Comparison	Effect size (z)	N
Fine *et al.* 2006	Tropical	Nutrients	Seedlings	Woody	Mono-terpenes	2	-0.19	114
Fine *et al.* 2006	Tropical	Nutrients	Seedlings	Woody	Sesqui-terpenes	2	-0.8	114
Fine *et al.* 2006	Tropical	Nutrients	Seedlings	Woody	Diterpenes	2	-0.36	114
Fine *et al.* 2006	Tropical	Nutrients	Seedlings	Woody	Total terpenes	2	-0.36	114
Folgarait & Davidson 1994	Tropical	Light	Seedlings	Woody	Total phenolics	2	-0.55	24
Folgarait & Davidson 1994	Tropical	Light	Seedlings	Woody	Condensed tannins	2	-0.55	24
Folgarait & Davidson 1994	Tropical	Light	Seedlings	Woody	Toughness	2	-0.55	24
Folgarait & Davidson 1994	Tropical	Light	Seedlings	Woody	food bodies	2	-0.55	24
Folgarait & Davidson 1994	Tropical	Light	Seedlings	Woody	food bodies	2	-0.7	24
Folgarait & Davidson 1995	Tropical	Nutrients	Seedlings	Woody	Nitrogen	2	-0.7	24
Folgarait & Davidson 1995	Tropical	Nutrients	Seedlings	Woody	Nitrogen	2	-0.7	24
Folgarait & Davidson 1995	Tropical	Nutrients	Seedlings	Woody	food bodies	2	-0.7	24
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Toughness	1	-0.47	25
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Toughness	1	-0.48	25
Hallam & Read,	Tropical &		Seedlings	Woody	Total phenolics	1	-0.54	25

Table S3.3. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Defense	Comparison	Effect size (z)	N
2006	Temperate							
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Tannins	1	-0.63	25
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Nitrogen	1	-0.5	25
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Nitrogen content	1	-0.6	25
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Protein content	1	-0.1	25
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Protein content	1	-0.09	25
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Water content	1	-0.26	25
Hallam & Read, 2006	Tropical & Temperate		Seedlings	Woody	Water content	1	-0.2	25
Holdo 2003	Tropical	Nutrients	Adults	Woody	Fiber	1	-0.09	1033
Howlett & Davidson, 2001	Tropical	Light	Seedlings	Woody	Toughness	1	-1.94	480
Massey *et al.* 2007	Temperate		Seedlings	Herbs	Silica concentration	2	-0.48	180
Massey *et al.* 2007	Temperate		Seedlings	Herbs	Abrasiveness	2	-0.47	180
Massey *et al.* 2007	Temperate		Seedlings	Herbs	Toughness	2	-0.21	180
Massey *et al.* 2007	Temperate		Seedlings	Herbs	Phenolic	2	-0.3	180
Massey *et al.*	Temperate		Seedlings	Herbs	Total defenses	2	-0.51	180

Table S3.3. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Defense	Comparison	Effect size (z)	N
2007								
Matsuki & Takayoshi 2006	Temperate		Seedlings	Woody	Total phenolics	2	-0.21	140
Matsuki & Takayoshi 2006	Temperate		Seedlings	Woody	Tannins	2	-0.04	140
Matsuki & Takayoshi 2006	Temperate		Seedlings	Woody	Toughness	2	-0.17	140
Matsuki & Takayoshi 2006	Temperate		Seedlings	Woody	Nitrogen content	2	-0.21	140
Matsuki & Takayoshi 2006	Temperate		Seedlings	Woody	Carbon content	2	-0.21	140
Miller & Woodrow 2008	Tropical	Nutrients	Adults	Woody	Nitrogen content	1	-2.04	96
Miller & Woodrow 2008	Tropical	Nutrients	Adults	Woody	Nitrogen content	1	-2.37	96
Newbery & de Foresta 1985	Tropical	Light	Seedlings	Woody	Phenolics	1	1.18	5
Newbery & de Foresta 1985	Tropical	Light	Seedlings	Woody	Tannins	1	0.39	5
Newbery & de Foresta 1985	Tropical	Light	Seedlings	Woody	Nitrogen content	1	1.87	5
Reich, *et al.* 1992	Temperate		Seedlings	Woody	Toughness	2	-0.85	287
Reich, *et al.* 1992	Temperate		Seedlings	Woody	Toughness	2	-0.69	287
Reich, *et al.* 1992	Temperate		Seedlings	Woody	Nitrogen content	2	-0.57	287
Reich, *et al.* 1997	Tropical & Temperate				Nitrogen content		-0.81	111

Table S3.3. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Defense	Comparison	Effect size (z)	N
Shure & Wilson 1993	Temperate	Light	Adults	Woody	Total phenolics	1	-0.39	240
Shure & Wilson 1993	Temperate	Light	Adults	Woody	Hydrolysable tannins	1	1.03	240
Shure & Wilson 1993	Temperate	Light	Adults	Woody	Condensed tannins	1	1.03	240
Shure & Wilson 1993	Temperate	Light	Adults	Woody	Toughness	1	-1.46	240
Shure & Wilson 1993	Temperate	Light	Adults	Woody	Water content	1	-1.15	240
Turner *et al.* 1993	Tropical	Light	Adults	Woody	Toughness	1	1.14	9
Turner *et al.* 1993	Tropical	Light	Adults	Woody	Toughness	1	2.54	10
Turner *et al.* 1993	Tropical	Light	Adults	Woody	Toughness	1	2.12	9
Turner *et al.* 1993	Tropical	Light	Adults	Woody	Toughness	1	-2.12	9
Turner *et al.* 1993	Tropical	Light	Adults	Woody	Toughness	1	-0.1	9
Turner 1995	Tropical	Nutrients	Adults	Woody	Total phenolics	1	1.09	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Total phenolics	1	0.97	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Total phenolics	1	1.09	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Total phenolics	1	1.89	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Total phenolics	1	1.13	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Total phenolics	1	1.6	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	1.9	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	-2.37	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	1.35	123

Table S3.3. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Defense	Comparison	Effect size (z)	N
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	-0.2	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	-0.62	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	0.78	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	-1.58	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	-0.99	123
Turner 1995	Tropical	Nutrients	Adults	Woody	Condensed tannins	1	-2.05	123
Turner *et al.* 2000	Tropical	Nutrients	Adults	Woody	Toughness	1	-0.54	27
Turner *et al.* 2000	Tropical	Nutrients	Adults	Woody	Toughness	1	-0.42	27
Turner *et al.* 2000	Tropical	Nutrients	Adults	Woody	Toughness	1	-0.36	27
Van Zandt 2007	Temperate	Nutrients	Adults	Herbs	Total defenses	2	-0.79	8

Table S3.4. Prediction (4) meta-analysis records for the effect of growth on herbivore damage. Comparison= Indicates whether the study was used for 1 (within-site) or 2 (among-site) sub-analyses; N= sample size.

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Comparison	Effect size (z)	N
Bryant *et al.* 1989	Tropical	Nutrients	Seedlings	Woody	2	1.68	30
Bryant *et al.* 1989	Tropical	Nutrients	Seedlings	Woody	2	0.97	30
Bryant *et al.* 1989	Tropical	Nutrients	Seedlings	Woody	2	1.28	30
Cebrian & Duarte 1994	Tropical & Temperate				1	0.5	56
Cebrian & Duarte 1994	Tropical & Temperate				1	1.18	56
Coley 1987	Tropical	Light	Saplings	Woody	2	0.29	47
Coley 1987	Tropical	Light	Saplings	Woody	2	0.58	47
Coley 1988	Tropical	Light	Saplings	Woody	2	0.58	41
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	2	0.44	60
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	2	0.11	60
Dalling *et al.* 2009	Tropical	Light	Seedlings	Woody	2	0.08	60
De la Cruz & Dirzo 1987	Tropical		Seedlings	Woody	1	-0.05	1450
Eichhorn, Fagan & Compton 2007	Tropical		Seedlings	Woody	2	1.29	5
Farji 2001	Tropical	Light	Adults	Woody	2	1.99	120

Table S3.4. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Comparison	Effect size (z)	N
Farji 2001	Tropical	Light	Adults	Woody	2	2.2	120
Holdo 2003	Tropical	Nutrients	Adults	Woody	2	0.77	27
Howlett & Davidson 2001	Tropical	Light	Seedlings	Woody	2	0.25	480
Lowman 1987	Tropical & Temperate		Adults	Woody	2	-2.39	80
Lowman 1987	Tropical & Temperate		Adults	Woody	2	-0.91	80
Lowman 1992	Tropical & Temperate		Adults	Woody	2	1.2	1672
Lowman 1992	Tropical & Temperate		Adults	Woody	2	-2.59	1672
Massey *et al.* 2007	Temperate		Seedlings	Herbs	2	0.56	180
Matsuki, & Takayoshi 2006	Temperate		Seedlings	Woody	1	0.15	140
McCanny *et al.* 1990	Temperate	Nutrients	Seedlings	Woody	1	-0.041	42
McCanny *et al.* 1990	Temperate	Nutrients	Seedlings	Woody	1	-0.005	42
Newbery & de Foresta 1985	Tropical	Light	Seedlings	Woody		-1.92	10
Richards & Coley 2007	Tropical	Light	Saplings	Woody	2	0.32	22
Shure & Wilson 1993	Temperate	Light	Adults	Woody	2	0.16	240
Skarpe *et al.*	Tropical	Nutrients	Adults	Woody	2	0.11	377

Table S3.4. (continued)

Reference	Ecosystem	Habitat Resource	Ontogeny	Functional Group	Comparison	Effect size (z)	N
2000							
Skarpe *et al.* 2000	Tropical	Nutrients	Adults	Woody	2	0.12	720
Skarpe *et al.* 2000	Tropical	Nutrients	Adults	Woody	2	0.12	720
Smith & Nufio 2004	Tropical	Nutrients			2	1.74	600
Southwood 1986	Temperate		Adults	Herbs & Woody	1	1.25	1400
Vasconcelos 1999	Tropical	Light & Nutrients	Adults & saplings	Woody	2	-0.48	581
Vasconcelos 1999	Tropical	Light & Nutrients	Adults & saplings	Woody	2	-0.94	559
Vasconcelos 1999 Wardle *et al.* 1998	Tropical Temperate	Light & Nutrients Nutrients	Adults & saplings Seedlings	Woody Herbs	2 1	0.18 0.46	114 20
Wardle *et al.* 1998	Temperate	Nutrients	Seedlings	Herbs	1	0.62	20
Wardle *et al.* 1998	Temperate	Nutrients	Seedlings	Herbs	1	0.78	20
Wardle *et al.* 1998	Temperate	Nutrients	Seedlings	Herbs	1	0.32	20
Wilf *et al.* 2001	Tropical & Temperate				2	1.07	2435

CHAPTER 3

DIVERGENT EVOLUTION IN ANTI-HERBIVORE DEFENSES WITHIN SPECIES COMPLEXES AT A SINGLE AMAZONIAN SITE

Abstract

Classic theory in plant-insect interactions has linked herbivore pressure with diversification in plant species. We hypothesize that herbivores may exert divergent selection on defenses, such that closely related plant species will be more different in defensive than in non-defensive traits. We evaluated this hypothesis by investigating two clades of closely related plant species coexisting at a single site in the Peruvian Amazon: *Inga capitata* Desv., and *Inga heterophylla* Willd. species complexes. We compared how these lineages differ in the suite of chemical, biotic, phenological and developmental defenses as compared to non-defensive traits that are related to habitat use and resource acquisition. We also collected insect herbivores feeding on the plants. Our data show that sister lineages within both species complexes are more divergent in anti-herbivore defenses than in other non-defensive, functional traits. Moreover, the assemblages of herbivore communities are dissimilar between the populations of coexisting *I. capitata* lineages. Taken together, our results are consistent with the idea that for the *I. capitata* and *I. heterophylla* species complexes, interactions with their natural enemies may have played a significant role in their phenotypic divergence and potentially in their

diversification and coexistence. It also suggests that defensive traits are evolutionary labile.

Introduction

The arms race between plants and insect herbivores has been invoked as one of the main mechanisms driving trait diversification and coevolution for both groups (Becerra 1997; Thompson 1998; Becerra 2009, Futuyma & Agrawal 2009; Kursar *et al.* 2009; Agrawal 2012). A fundamental prediction of this theory is that herbivores drive the evolution of plant anti-herbivore defenses faster than for other traits (Thompson 2005; Kursar *et al.* 2009). Testing this hypothesis requires demonstrating that sister species are more different in anti-herbivore defenses than in traits related to adaptations to other extrinsic factors, such as the abiotic environment. However, studies testing this idea are surprisingly few (Agrawal 2009b). Consequently, in this study we combine data on plant functional traits and insect herbivores to compare patterns of divergence in two groups of closely related species coexisting at a single site.

The coevolutionary theory of plant-herbivore interactions suggests that the production of defenses against insects has played a dominant role in host and enemy radiations (Ehrlich & Raven 1964). Specifically, this theory predicts a tight correlation between plant relatedness and plant defenses. Although widely accepted, relatively few studies have tested this, and some even question the fundamental assumptions of this theory. For example, Becerra (1997) found only a weak relationship between the phylogenetic hypothesis and chemical similarity for the species of *Bursera,* common trees in the dry forests of Mexico. Likewise, Kursar *et al.* (2009) found a weak

correlation between phylogenetic distances and chemical distances within the Neotropical tree genus, *Inga.* This lack of phylogenetic signal in the expression of secondary metabolites suggests divergent selection on anti-herbivore defenses, such that closely related species are not necessarily similar in defenses. This should make it more difficult for herbivores to track hosts over evolutionary time thereby reducing herbivore pressure on plants.

Although the role of the physical environment on trait divergence has received considerable attention (Anacker & Strauss 2014), the role of defensive traits in plant diversification is not well understood (Futuyma & Agrawal 2009). Yet, several studies have provided indirect evidence for the significance of defensive traits in evolutionary diversification by showing a relationship between variation in these traits and species diversity (Farrell *et al.* 1991; Becerra 1997; Agrawal & Fishbein 2008; Agrawal *et al.* 2009a; Agrawal *et al.* 2009b; Kursar *et al.* 2009). A better understanding of the relative importance of defensive traits in phenotypic diversity and species divergence will require examining differences in defensive and non-defensive traits simultaneously between recently diverged species or populations at an incipient state of divergence (Agrawal & Futuyma 2009; Fine *et al.* 2013).

Here, we examine the contribution of plant-insect interactions to divergence among species by determining variation in functional traits and herbivore communities within two clades of closely related species coexisting at a single site in the Peruvian Amazon: *Inga capitata* Desv. and *Inga heterophylla* Willd. species complexes. The taxa in each complex are considered a single species based on the morphological traits of reproductive individuals (Pennington 1997). Our field observations of subtle differences

within each clade in color of the expanding leaves (see Appendix A.1), number of leaflets and stipule morphology have motivated the present characterization of trait divergence within these clades. In fact, plastid DNA analyses distinguish each member as a different evolutionarily significant unit (ESU) and as sister taxa (Kursar *et al.* 2009, Appendix A.2). Based on these analyses, *Inga capitata* comprises three ESUs and *Inga heterophylla* two ESUs. Four of the ESUs co-occur in terra firme habitats, often within meters of each other, whereas one *I. capitata* ESU species (cap2) occurs primarily in the nearby floodplains.

In order to achieve a comprehensive analysis, we collected data on many different defensive traits including chemical, biotic, phenological and developmental defenses, as well as on insect herbivores. We also collected data on non-defensive traits that are related to habitat use and resource acquisition. Our previous studies on the genus *Inga* suggest that defenses evolve rapidly (Kursar *et al.* 2009). Specifically, we expect the ESUs within an *Inga* lineage to be more similar with respect to non-defense traits such as primary metabolites and resource acquisition traits. In contrast, the observation that anti-herbivore traits show a greater difference among relatives than for non-defense traits would support the key role of herbivores in shaping divergence and niche separation in their host plants.

Methods

Study site

This study was conducted at Los Amigos Biological Station (Spanish acronym: CICRA, Centro de Investigación y Capacitación Rio Los Amigos). Los Amigos is located

in the south-eastern Peruvian Amazon, in the Madre de Dios Department at 12°34'9"S, 70°6'0.40"W, 268 m.a.s.l. Los Amigos covers 453-ha of lowland Amazonian forest and consists of a mosaic of terra firme and floodplain forests. Mean annual rainfall is between 2700-3000 mm, and the mean monthly temperature ranges from 21 - 26°C (Pitman 2007).

Study species

Inga capitata comprises three phenotypically divergent ESUs: cap1, cap2 and cap3 (Kursar *et al.* 2009). In addition, they present different habitat preferences, with cap1 and cap3 showing a preference for terra firme and cap2 for floodplains. The *I. heterophylla* species complex includes two phenotypically different lineages: het1 and het2 (Kursar *et al.* 2009), both on terra firme. For those ESUs found in terra firme, one ESU often is meters away from another and no intermediates were observed. The study plants were widely distributed within their respective habitats types; aside from as noted above, inspection of the location data and our and our field observations showed no tendency for the study species to be clumped or restricted to certain habitats (e.g., preference for treefall light gaps).

Censuses and leaf traits

In the present study, anti-herbivore defenses are defined as those plant traits that have been selected in response to herbivory. These include developmental defenses (leaf expansion rate Kursar & Coley 2003), biotic defenses (leaf-defending ants and the area of extrafloral nectaries ; Koptur 1984; Brenes-Arguedas *et al.* 2008), phenological defenses

(the timing and synchrony of young leaf production, Aide 1993; Kursar & Coley 2003), and chemical defenses (phenolics and non-protein amino acids; Coley *et al.* 2005). This set of defense traits was measured only on expanding leaves because more that 80% of the damage accrued during a leaf's lifetime happens during the short period (1-3 weeks) of leaf expansion (Coley & Aide 1991; Kursar & Coley 2003; Brenes-Arguedas *et al.* 2006). Therefore, young-leaf defenses are under strong natural selection by herbivores.

Traits under selection from the physical environment are considered here as non-defense traits. These traits were measured only on mature leaves. These include leaf mass per area (LMA), leaf nitrogen content, area per leaflet, number of leaflets per leaf, and the presence or absence of wings. These include some of the key ecophysiological attributes that correlate with photosynthetic capacity and transpiration, with habitat type such as light availability and with resources such as soil nutrient content (Cornelissen *et al.* 2003; Wright *et al.* 2004; Fujita *et al.* 2013). Although, in principle, the LMA and leaf nitrogen of mature leaves can affect leaf palatability to herbivores, in actuality, herbivores attack the mature leaves of shade-tolerant tropical rainforest plants, such as *Inga*, at low rates. Consequently, we consider that LMA and leaf nitrogen of mature leaves are more important as adaptations for resource acquisition and habitat and not to herbivore pressure (Endara & Coley 2010).

Data were collected for young and mature leaves on 0.5-4 m tall saplings in the shaded understory from 2007 until 2011. More than 100 km of trails were walked regularly to search for plants and collections were widely separated. Specifically, based on trail locations, we estimate that, for each ESU, our collections were made, on average, every 360 m. Leaf expansion rate was quantified for leaves between 20% and 80% of full

size by measuring their area every 1-4 days until they were fully expanded. To quantify synchrony in leaf production for each ESU in the *I. capitata* complex, 30-70 individuals per ESU were marked and each plant was scored monthly for the presence of young leaves. *I.* cap1 was censused between June-December of 2010. *I.* cap2, *I.* cap3 and *I.* het1 were censused between January-December 2007. Due to the low abundance of *I. heterophylla* het2 saplings, it was not possible to measure synchrony in leaf flushing or the following leaf traits. At each census, the number of ants visiting the extra-floral nectaries of expanding leaves was quantified (# of ants per nectary). The area of the nectary was estimated using a dial caliper. Leaf mass per unit area (LMA; g m^{-2}) was measured from discs of mature leaves of known area that were dried at approximately 70 °C for 3 days. Mature leaves were ground and analyzed for leaf N content with a Costech 4010 Elemental Analyzer coupled to a Thermo Delta Plus XP IRMS (Costech Analytical Technologies). The number and size of leaflets were calculated for at least three leaves per sampled individual and the presence of wings on the rachis was recorded.

Chemical analysis

Metabolites were extracted, separated, quantified gravimetrically, and analyzed using ultra high performance liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (LC-MS and GC-MS, respectively). Expanding leaves from five individual understory saplings were collected for each ESU of *I. capitata* and *I. heterophylla*. For each individual sapling we collected expanding leaves that were 80% of the average maximum size. Fresh leaves were dried in silica gel at room temperature and shipped to the University of Utah for chemical analysis. Only soluble metabolites

were studied; thus, those covalently bound to cell walls were excluded (Lokvam & Kursar 2005).

Extract preparation

The protocol of Bixenmann *et al.* (2013) was followed with some modifications. For each sample, 300 – 500 g of vacuum-dried leaf material was homogenized using a ball mill (Retsch, MM 200, Haan, Germany) at 30 Hz for 30 s. Approximately 100 mg of each homogenized plant sample was weighed in an Eppendorf tube and mixed with 15.0 microliters of a 1 mg/mL amino acid internal standard solution (a mixture of 20 amino acids labelled with 13C and 15N; Sigma-Aldrich) and 1.5mL of 70% acetonitrile 30% water (v/v). We extracted with 70% acetonitrile (acetonitrile:water, 70:30, v/v) instead of ethanol, a typical solvent used for extraction, because we found that polygallate esters are unstable in ethanol (data not shown). After extraction for 10 min and centrifugation (13,793 x g) for 5 min, the supernatant was transferred to a glass vial and the extraction repeated for a total of three times. The extraction was repeated two more times using 1 mL of 70% acetone (acetone:water, 70:30, v/v). The extracts were combined and dried under nitrogen gas until all organic solvents were evaporated. To remove lipids, 3 mL of water and 3 mL of hexane were added to the dried extract. After vortexing for 5 s, the extract was left to settle for a few minutes until two distinct layers formed. The non-polar fraction was then transferred to another pre-weighed glass vial and the extraction repeated with 3 mL of hexane. Both the non-polar and the polar organic fractions were dried under nitrogen gas and then under vacuum (0.8 torr) at ambient temperature.

The polar organic fraction was separated on an Octadecylsilane (ODS; 40 μm Prep LC Packing) column. ODS (2.9 g) was dry-packed in a 10 mL syringe. The dried extract was suspended in 2 mL of water and transferred to the ODS column. Thirty mL of water was run through the column and collected in a pre-weighed glass vial (polar fraction). This process was repeated with 50% acetonitrile 50% water (v/v), followed by 100% acetonitrile to collect the phenolic and saponin fractions, respectively. After removal of solvents and vacuum drying (0.8 torr) at ambient temperature, each fraction was weighed. The weight for the saponin fraction was negligible and is not considered further.

GC-MS analysis

The water or polar fraction was analyzed by GC-MS using a GCT Premier mass spectrometer (Waters, Milford, MA, USA) fitted with a GC6890 gas chromatograph (Agilent Technologies) and a Gerstel MPS2 autosampler (Gerstel, Mülheiman der Ruhr, Germany). The dried polar fraction (0.25-0.47 mg) was suspended in 40 μL of 40 mg mL^{-1} O-methoxylamine hydrochloride in pyridine and incubated for one hour at 30°C. To this solution, 25 μL of N-methyl-N-trimethylsilyltrifluoracetamide was added using the autosampler and incubated for 30 min at 37°C with shaking. One μL of the sample was injected in to the gas chromatograph at a 10:1 split ratio with the inlet temperature held at 250°C. The gas chromatograph had an initial temperature of 95°C for 1 min followed by a 40°C min^{-1} ramp to 110°C and a hold time of 2 min. This was followed by a second 5°C min^{-1} ramp to 250°C, a third ramp to 350°C, then a final hold time of 3 min at

350°C. A 30 m ZB-5MSi column (Phenomenex, Torrance, California, USA) with a 5 m guard column was employed for chromatographic separation.

LC-MS analysis

Liquid chromatography was performed on an I-Class Acquity Ultra Performance Liquid Chromatography system (Waters, Milford, MA, USA). Dried phenolic extract (0.01-0.055 mg) was resuspended in 1 mL of 50% acetonitrile 50% water (v/v), centrifuged (13,793 x g) for 5 min, and the supernatant transferred to a HPLC vial. One µL of sample was injected on an Acquity UPLC BEH C18-column (50mm x 2.1 mm x 1.7um) (Waters, USA). Sample and column temperatures were held constant at 10°C and 40°C, respectively. Samples were eluted using a mobile phase of 0.3 mL min^{-1} with the gradient shown in Appendix A.3. The mobile phases consisted of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). The solvents are Fisher LC-MS grade.

Compounds were detected using a Xevo G2 QToF mass spectrometer (Waters, Milford, MA, USA) equipped with a lock spray and an electrospray ionization source (ESI). Spectra were collected in positive ionization mode (ES+) in the *m/z* range of 50 to 1200. The parameters of the ESI were: capillary voltage of 2.3 kV, sampling cone voltage of 30V, extraction cone voltage of 4 V, source temperature of 120°C, desolvation gas temperature of 400°C, desolvation gas flow of 900 L hr^{-1} and collision energy of 6 eV. The mass spectrometer was calibrated using a sodium formate standard (0.5 mM in 90% 2-propanol 10% water (v/v)), and leucine enkephalin (2 ng/µL) was used as a lock mass.

Insect herbivores

To assess whether differences in defensive traits between the different ESUs relate to differences in herbivore choice, we performed: a) a field survey of the abundance of leaf chewing insects feeding on expanding leaves of the *Inga capitata* species complex and b) a captive choice experiment with sawfly larvae (*Symphyta*, Argidae) that only fed on this species complex and was its most abundant herbivore. For the herbivore survey, all leaf-chewing insects that were found feeding on the expanding leaves of saplings in the understory were recorded. Insects were collected by hand from the leaves between 2010 and 2011 for a period of 10 months, as part of a project that is examining the insect herbivore fauna feeding on the entire genus *Inga* in Los Amigos. Each plant was visited once every flush. All Coleoptera were classified to genus based on morphology by specialists associated with the Pontificia Universidad Catolica del Ecuador. All lepidopteran and sawfly insects were assigned to MOTUS (Molecular Operational Taxonomic Units) using COI sequences. PCR amplification and DNA sequencing were generated at the Canadian Center for Barcoding, and in our laboratory using standard barcoding protocols (Ivanova 2006; deWaard 2008). PCR amplification with either the LCO/HCO or LepF1/LepR1 primer pairs recovered a 658 bp region that was subsequently used to generate MOTUS.

For the feeding choice experiment, sawfly larvae and expanding leaves of the three *I. capitata* ESUs were collected in the field. In the laboratory, leaves were cut into square pieces of approximately 39 cm^2. Sawfly larvae were deprived of food overnight prior to the experiment and then three pieces of leaf (one per ESU) were offered to an individual larva (N=9). The experiment was carried out in Petri dishes lined with

moistened filter paper. After 24 hours, the area eaten on each square leaf piece was recorded using an acetate grid.

Data analysis

Leaf traits

The censuses were analyzed for synchronization in leaf production for each ESU using circular statistics (Zar 1999). Months were converted to angles between 0° and 360°. The vector length r was calculated for each population following Zar (1999). The length of the vector *r* varies between 0 and 1, and is a measure of seasonality. High values of *r* indicate aggregated phenological behavior, and low values represent a uniform distribution of phenological activity throughout the year (Batschelet 1981). We determined whether ESUs differed in their season of leaf production using the Watson's test (U2) (Zar 1999).

Differences among ESUs in the number of ants visiting the extrafloral nectaries, nectary area, leaf expansion rate as percent increase in area per day, leaf mass per area, number and size of leaflets, and phenolic contents were examined using Analysis of Variance (ANOVA) followed by Tukey *post hoc* tests for multiple comparisons. Tests for normality of the data, and appropriate data transformations were performed. These analyses were performed in the statistical programming language R version 3.0.1 (R Development Core Team 2011).

Chemical traits

Raw data from the GC-MS and the UPLC-MS were processed for peak detection and peak alignment using MarkerLynx (MassLynx v 4.1 Waters, Manchester, UK) and XCMS (Smith *et al.* 2014). The output files from the UPLC-MS were further processed for data quality as follows: Because no late eluting saponins were found, the retention time window of interest was delimited to 22 min (peaks at >22 min to the end were discarded). Peaks (often referred to as "features") that were not consistently detected were discarded. For this, all peaks that occurred in only one species and in three or fewer replicates of that species were discarded. Zero or missing values were replaced with half of the minimum positive value in the dataset. Because our dataset contained a large number of variables (>490 peaks), multiple hypotheses were tested for each peak. Hence, we applied a filtering method in order to adjust for multiple testing (Hackstadt & Hess 2009). As recommended in Metaboanalyst (see below) for a sample size of about 500 peaks, we eliminated the 10% of peaks with the lowest intensities. For this, peak intensities were ranked based on the Interquantile Range. Peak intensities, or the total ion current, were normalized by the dry weight of the sample. The most important compounds for discriminating metabolic differences between these three ESUs, or "biomarkers" were tentatively identified based on MS/MS or as unknowns that were classified based on retention time plus the mass to charge ratio (*m/z*, Appendix A.6). Unknowns from the GC-MS analysis were compared to the NIST database version 2.0 (2005) containing approximately 30,000 compounds.

To quantify metabolite-wide variation among *Inga* ESUs, multivariate statistical methods were used. First, normalized peak intensities were Pareto scaled. Subsequently,

a PCA model, a PLS-DA model, and hierarchical clustering were fitted on the scaled data in order to see grouping patterns. When the PCA model was non-significant (no clustering) the PLS-DA and hierarchical clustering analyses were not performed. The hierarchical clustering was performed using the Pearson's correlation similarity measure and the Ward's linkage clustering algorithm. All metabolomic data analyses were performed using the Metaboanalyst webserver (Xia *et al.* 2009).

Insect herbivores

COI sequences of sawflies and lepidopterans were assembled into contigs and manually edited using the program Sequencher v 5.1 (Gene Codes). The resulting sequences were subsequently aligned using the program MUSCLE (Edgar 2004), and clustered into MOTUS using the software package jMOTU (Jones *et al.* 2011). Then, the abundance and composition of these MOTUS were compared among the *I. capitata* ESUs using multivariate analyses. All the feeding records that were limited to a single individual in a particular host were not included in this analysis. For this reason, from 64 plants and 37 herbivore species that were originally sampled, only 38 plants and nine herbivore species were included in the analysis. Overlap in feeding records was estimated using the Bray-Curtis dissimilarity index with standardized raw data. The resulting matrix was then analyzed for differences in herbivore communities between ESUs using a two-dimensional non-metric multidimensional scaling (NMDS) ordination and a permutational multivariate analysis of variance (PERMANOVA, Anderson 2001) with adjusted p values following the Holm-Bonferroni correction for multiple comparison testing. These analyses were performed using the R package vegan (Oksanen 2013).

Host selection and feeding preferences for the sawfly larvae in the field and in the laboratory were estimated using a hierarchical Bayesian model designed for count data (Fordyce *et al.* 2011). This analysis was performed using the R package bayespref (Fordyce *et al.* 2011) in a Markov Chain Montecarlo (MCMC) framework. Two models were run to estimate the strength of preference of the sawflies for a particular ESU. One model was run with individuals constrained to have equal preference for the different ESUs, and the other model was developed with variation in preference among ESUs (unconstrained). The DIC (deviance information criterion) value was used to compare the fit of each model. In both models, the MCMC chains were run for 5,000 generations with the first 1,000 generations discarded as a burn-in. Significant differences between ESUs were analyzed using pair-wise comparisons of the proportion of times that a sawfly had a greater preference parameter for a particular ESU at each step of the MCMC.

Results

Inga capitata species complex

Leaf defense traits

Consistent with the color and morphology of expanding and mature leaves, and with DNA sequence differences, each ESU had distinct defensive traits, with no intermediates observed. Total production of soluble phenolics in expanding leaves ranged from 32% to 39% of leaf dry weight across the three ESUs (Appendix A.4), values that are typical for the genus as a whole. The fact that these metabolites had substantial detrimental effects in the lab at only 0.5% to 2% of diet (Coley *et al.* 2005; Lokvam & Kursar 2005; Lokvam *et al.* 2006) demonstrates that the in vivo level of circa 35% must

be highly toxic. The profiles of defensive metabolites showed clear qualitative differences among ESUs (Appendix A.5). Quantitative analyses of the UPLC-MS metabolomics data (phenolics) for clustering showed complete separation among the three *I. capitata* ESUs, with cap3 being the most distinct (Fig. 3.1). Similarly, a PLS-DA analysis of the metabolites within the phenolic fractions in the UPLC-MS clearly separated cap1, cap2 and cap3 (Fig. 3.2a). Component 1 separates cap3 from cap1 and cap2, whilst the second component illustrates the clear contrast between cap1 and cap2 (R^2= 0.8, P< 0.05, Fig. 3.2a). The most important biomarkers for discriminating metabolic differences between these three ESUs were a series of tyramine gallates and quinic acid gallates that are relatively more abundant in cap3. The chemistries of cap1 and cap2 were distinguished from each other and from cap3 by a series of unknowns and kaempferol-galloyl-hexose (Appendix A.6).

Species of *Inga* also produce highly polar secondary metabolites such as toxic non-protein amino/imino acids that are isolated in the polar fraction and have been shown to have a toxic effect on herbivores (Coley *et al.* 2005; Lokvam *et al.* 2006). Metabolomic analysis of the secondary metabolites in the polar fraction included non-protein amino acids that were identified using standards (such as L-DOPA, β-alanine, homoserine, hydroxyproline) and uncharacterized compounds that are thought to be secondary metabolites because they were abundant and did not match any of the more than 30,000 known small molecules in the referenced databases. This also showed a trend for differences among the ESUs (R^2= 0.8, P= 0.06, Fig. 3.2b).

The timing and frequency of leaf production differed considerably between the ESUs within the *Inga capitata* species complex (Fig. 3.3). Patterns of leaf flushing

between cap2 and cap3 were significantly different ($P \leq 0.01$). ESU cap1 was not compared statistically as data were collected in a different year and for only 7 months. Low population vector lengths for cap2 and cap3 indicated low synchronization in leaf production (cap2 $r= 0.22$, cap3 $r= 0.26$), although relative peaks in leaf production were observed in July and October, respectively. In contrast, cap1 had a peak in September (of a different year) and, although several censuses were missed, a large population vector length ($r= 0.8$) for cap1 indicated a high degree of synchrony in leaf production.

The three ESUs differed also in the average area of extrafloral nectaries ($F_{2, 140}= 47.75$, $P< 0.01$, Fig. 3.3). Ant visitation to extrafloral nectaries of *I. capitata* saplings differed among the three ESUs ($F_{2,84}= 5.71$, $P< 0.05$, Fig. 3.3). Ant abundance on cap1 and cap3, the terra firme ESUs, was two times higher than on cap2 ($P< 0.05$), the floodplain ESU. Another strategy to reduce the impact of herbivory is to expand leaves rapidly, which minimizes the period of greatest vulnerability to herbivores (Kursar & Coley 2003). We found that the three ESUs differ dramatically in the rate of leaf expansion, with cap1 showing a significantly higher percentage increase in leaf area per day than cap2 and cap3 ($F_{2,16}= 33.29$, $P< 0.01$, Fig. 3.3).

Insect herbivores

Consistent with the observed differences in defensive traits, the abundance and composition of the insect herbivore assemblages showed divergent patterns between the *I. capitata* ESUs (Full model $F_{2,36}= 3.16$, $P< 0.01$; cap1 vs cap2 $F_{1,22}= 2.37$, $P<0.05$; cap1 vs cap3 $F_{1,24}= 2.09$, $P<0.05$; cap2 vs cap3 $F_{1,26}= 1.77$, $P<0.05$, Fig. 3.4). The ordination diagram showed separation among the three ESUs, with cap1 supporting the most

distinctive herbivore fauna. The permutational analysis of variance suggested that ESU is a more important factor than habitat in explaining the variation associated with the host selection by herbivores (ESU R^2= 0.2, P<0.01, habitat R^2= 0.007, P<0.05). Given that there are three ESUs we also performed a more restricted analysis that included only those herbivore species that were collected three or more times. No conclusions were affected, except that the differences between ESUs were more significant (Full model: P<0.001, ESU R^2= 0.25, P<0.01, habitat R^2=0.005, P<0.05, Appendix A.8).

Results from our choice experiment suggested that, even for a shared herbivore species, differences in chemical defenses within the *I. capitata* complex are big enough to affect herbivore preference. While the sawflies were found on all *I. capitata* ESUs in the field, they showed a significantly higher preference for cap2 over cap1 and cap3, both in the field and in the choice experiment (pairwise post-burning comparisons, P < 0.05 for all comparisons between cap2 and the two other ESUs, Fig. 3.5, Appendix A.9).

Leaf non-defense traits

Five non-defensive, functional traits were measured. Leaf mass per area (LMA) and leaf nitrogen content are widely used indicators of habitat specialization and photosynthetic ability. Both measures did not vary across ESUs (LMA: $F_{2,12}$= 2.25, P=0.15, nitrogen: $F_{2,12}$= 2.77, P=0.1, Fig. 3.6). The presence vs. the absence of wings on the rachis also did not show significant differences between ESUs (Fig. 3.6). Similarly, GC-MS analysis showed that primary metabolites in the polar fraction, such as protein amino acids, did not differ. The PCA model fitted to the primary metabolite data did not reveal separate clusters for any of the ESUs (Fig. 3.7a) neither for the first two principal

components, nor for any other combination of components. However, saplings of cap1 had smaller leaflets ($P< 0.01$) and fewer leaflets per leaf ($P< 0.01$) than cap2 and cap3 (Fig. 3.6).

Inga heterophylla species complex

Leaf defense traits

The two ESUs within the *I. heterophylla* complex are extremely different from each other with respect to their phenolic compounds (Appendix A.5), with het1 showing the greatest divergence (Fig. 3.1). In fact, *I. heterophylla* het2 more closely groups with *I. capitata* cap1 and cap2. For het1 and het2, total phenolic investment varied between 17% and 23% of leaf dry weight (Appendix A.4). Compounds detected within the phenolic fraction in the UPLC-MS clearly separated het1 and het2 by the first axis ($R^2= 0.9$, $P< 0.01$; Fig. 3.2c). Saplings of het1 are distinguished from het2 by the expression of relatively high abundant markers tentatively identified as tyrosine gallate, a class of compounds only known from *Inga* (Lokvam *et al.* 2007), and galloyl-L-DOPA (Appendix A.6). Analyses of the non-protein amino/imino acid fraction also separated the two ESUs ($R^2= 0.9$, $P< 0.05$; Fig. 3.2d), with the primary differences being high levels of free tyrosine and L-DOPA in het1. No marker phenolics or amino/imino acids were found in het2. An insufficient number of individuals of het2 did not enable us to perform statistical analyses for the other defensive traits.

Leaf non-defense traits

The two non-defensive traits for which we had sufficient data did not differ. Neither ESU had wings. The metabolic fingerprint of primary metabolites did not discriminate between het1 and het2 (Fig. 3.7).

Discussion

Divergence in defenses

Insect herbivores are predicted to be major selective agents (Agrawal *et al.* 2012) and results from our analyses are consistent with this idea. First, we found a substantial investment in plant defenses against herbivores. Total soluble phenolics accumulated to 32% to 39% of the dry weight of leaf tissue for the *I. capitata* species complex, and to 17% to 23% for the *I. heterophylla* group. In addition, both groups invest in other costly defenses, such as non-protein amino acids, extrafloral nectar production, and phenological and developmental defenses. Second, the factors with the highest divergence between closely related ESUs, for both species complexes, were the anti-herbivore traits. And thirdly, for the *I. capitata* complex, close relatives were attacked by different insect herbivore assemblages. Taken together, these results are consistent with the hypothesis of strong selection on defenses by herbivores, and suggest that these traits are evolutionarily labile (Agrawal 2008; Kursar *et al.* 2009; Schemske 2009).

Among all defensive traits, the most contrasting and interesting differences were found in chemistry. Although all defenses are important, clearly chemistry plays a central role in plant-herbivore interactions (Thompson 1988). The metabolomic analyses provided evidence for divergence in secondary metabolite expression (phenolics and non-

protein amino acids) for each species complex by separating the different ESUs and identifying ESU-specific "bio-markers" (Figs. 3.1, 3.2 and 3.7, Appendix A.6).

As with secondary metabolites, phenological defenses diverged markedly between the *I. capitata* lineages (Fig. 3.3). Synchronous production of leaves is a strategy to satiate herbivores because, by flushing leaves simultaneously, leaf biomass production may exceed the capacity of insects to consume them; this is considered a phenological defense (Aide 1988; 1993). In addition, because in tropical forests the temporal peak in leaf consumption by insect herbivores closely tracks leaf production (Murali & Sukumar 1993), especially for the most synchronous plant species (Lamarre *et al.* 2014), temporal separation of leaf production among species may be favored as a strategy for partial escape from herbivores. Individuals of cap1 showed a greater synchrony in leaf production than cap2 and cap3 (Fig. 3.3). In addition, timing for leaf production was different between ESUs, that is, during the study period, the main peaks of leaf production were at different times of the year, with September for cap1 (data collected in a different year), July for cap2, and June for cap3. Young leaves are an ephemeral stage in the life of a leaf that lasts only a few weeks. Thus, time lags of only two weeks between ESUs would be biologically meaningful with different ESU's being available for oviposition at different times of the year (Aide & Londoño 1989).

Similarly, our analyses found differences among ESUs in the rate at which young leaves expand, with leaves from cap1 expanding significantly faster than leaves from cap2 and cap3 (Fig. 3.3). Shortening the window of vulnerability to herbivores provides a mechanism for temporal escape (Aide & Londoño 1989). It appears that the strategy of escaping herbivory by expanding leaves rapidly is fueled by delaying the development of

the chloroplast (delayed greening) until the leaf is fully expanded and defended by toughness (a defense syndrome termed "escape species," see Kursar & Coley 2003). Although delayed greening reduces the photosynthetic capacity of young leaves, it also reduces the resources that are lost per gram of leaf eaten (Coley & Kursar 1996).

Biotic defenses also diverge between ESUs in the *I. capitata* group. Our field observations indicated that, although all the three lineages invest in active extrafloral nectaries, they differ in the area of the nectary (a proxy for the amount of nectar production, Rudgers 2004; Diaz-Castelazo *et al.* 2005) and in the number of ants visiting each ESU (Fig. 3.3). Ant visitation to the two terra firme ESUs, cap1 and cap3, mirrors differences in extrafloral nectary size. Expanding leaves of cap1 received less ant visitation than leaves from cap3. This pattern could result from more nectar production in cap3, as ants respond positively to higher concentrations and volume of nectar (Bixenman *et al.* 2011). However, although cap2 has extrafloral nectaries that are intermediate in size, it received significantly lower ant visitation than the other two ESUs, presumably because it occurs in flooded forests where the abundances of ground-nesting ants might be lower (Pearson & Derr 1986).

In contrast to what we found with defensive traits, leaf functional traits that are unrelated to defense showed less variation between closely related ESUs. Neither species complex differed in the expression of primary metabolites (Fig. 3.7). Similarly, leaf mass per area and nitrogen content of mature leaves did not show significant differences between the ESUs of the *I. capitata* complex (Fig. 3.6). These findings suggest that adaptations to the abiotic environment, such as light and nutrients, may not have acted as drivers of divergence between closely related ESUs. However, cap1 did differ in the size

and number of leaflets from cap2 and cap3 (Fig. 3.6), with cap1 having only four leaflets of consistently smaller size, and cap2 and cap3 having between four and six larger leaflets. Given that adults from these three ESUs show a reduction in leaflet size and mostly four leaflets per leaf (Pennington 1997, MJ Endara pers. obser.), leaf morphologies may differ only at the sapling stage. The fact that cap2 occurs in the floodplain suggests that adaptations to seasonal flooding might be an important factor in divergence between this ESU and the terra-firme ESUs, cap1 and cap2. Although we did not measure plant traits associated with flooding tolerance, quantitative trait-based studies have found that species adapted to flood-prone environments show higher LMA and greater leaf area than species from other habitats (Colmer & Volsenek 2009; Huber *et al.* 2009). In our study, these traits did not differ between the terra-firme and floodplain ESUs.

We also did not find distinct microhabitat preferences of the terra firme forms. ESUs cap1 and cap3, the shade-tolerant, terra-firme ESUs coexist within meters of each other, similar to *I. heterophylla* ESUs het1 and het2. Moreover, all five ESUs occur in low-light microsites and show no preference for treefall light gaps.

Studies in other lineages have also suggested greater divergence among closely related species between defensive as compared to non-defensive traits. In the milkweeds, a significant correlation between variation in defensive traits and diversification has been found, while other, non-defensive traits did not show such a relationship (Agrawal *et al.* 2009b). Likewise, close relatives of the genus *Psychotria* (Rubiaceae), an understory shrub of tropical forests, are more dissimilar in secondary metabolites than in traits associated with shade and drought tolerance (Sedio 2013). These findings suggest that

defenses are relatively more labile than other traits, and highlight the importance of insect herbivores in trait diversification.

Divergence in insect herbivore selection

Consistent with the observed differences in defensive traits, the abundance and composition of insect herbivore communities showed divergent host association between the *I. capitata* ESUs (Fig. 3.4). It is quite striking that these differences are consistent even when considering the whole *Inga* community in the study area (43 *Inga* species). Dissimilarities in Lepidoptera community between two of the three pairs of *I. capitata* ESUs are significantly greater than between more distantly related *Inga* species. The community mean dissimilarity index β_{RC} equals 0.69, whereas for cap1 vs cap2 β_{RC} equals 0.74 and for cap2 vs cap3 β_{RC} equals 0.92. But for cap1 vs cap3 β_{RC} equals 0.10 (two-tailed test, alpha=0.05, unpublished analyses of MJE; for β_{RC} analyses see Chase *et al.* 2011). Similarly, Fine *et al.* (2013) found substantial differences in secondary metabolites and in the abundance and diversity of insect herbivores between two ecotypes of *Protium subserratum* that occur in white-sand and terra firme forests in the Peruvian Amazon. In our study, the two *I. capitata* lineages (cap1 and cap3) that occur within the same habitat, terra firme, showed the biggest difference in total herbivore assemblage (including Coleoptera, sawflies and Lepidoptera, Fig. 3.4). This may result from the fact that, in terms of phenolic composition, cap1 more closely groups with cap2 than with cap3 (Fig. 3.1). Taken together, these results suggest that herbivores might select for divergence in defenses among coexisting lineages.

Our feeding-choice experiment and field survey of sawfly larvae both showed a preference for cap2 over the other two ESUs. This suggests that differences in chemical defenses within the *I. capitata* complex are big enough to affect herbivore preference, even for those herbivore species that are shared. Although many factors can influence host selection in the field, including habitat preferences, phenology, and ant visitation to extrafloral nectaries, the primary factors assessed in our controlled choice experiment were leaf secondary metabolites and possibly nutrition. This consideration and the observation that, for sawfly larvae, host selection is related to plant phenolics (Opitz *et al.* 2012) are consistent with our bioassay results.

Patterns in defense divergence

Although both clades include closely related ESUs, two patterns of divergence in chemical defenses are evident, with one species complex being more divergent than the other (Fig. 3.1). One trend is exemplified by the *I. heterophylla* complex, where the two ESUs express non-overlapping chemistry (Fig. 3.1 and Appendix A.6). A switch in secondary compounds between sister species is often found across the genus *Inga* (Kursar *et al.* 2009). Similar results have been obtained for the Fabaceae in a recent study (Wink 2013), and for other groups, such as *Bursera* (Becerra 1997). These patterns diverge from the dominant paradigm of defense evolution, which predicts that closely related species have similar defenses (Ehrlich & Raven 1964), and suggests that the production of novel defense mechanisms arise primarily through stepwise changes to structural genes coding for novel biosynthetic enzymes (Berenbaum & Feeny 1981; Berenbaum & Zangerl 1998; Berenbaum & Schuler 2010). We speculate that large shifts in defense chemicals between

sister species can be better explained through changes in gene regulation rather than in structural genes for biosynthetic enzymes. In fact, most studies of systems at the genetic level report that chemical traits have diverged due to changes in regulation (Durbin *et al.* 2003; Tewari *et al.* 2003; Windsor *et al.* 2005; Burow *et al.* 2010).

The second pattern in defense divergence is also consistent with regulatory changes. In the *I. capitata* complex, the three lineages within the group show related chemistry. The most common compounds expressed by the three ESUs are derived from the same pathways, with differences between close relatives found mainly at the level of expression of the different metabolites and/or structural complexity. For example, tyramine gallates, biomarkers for cap3, occur across the three ESUs. However, its relative abundance is much higher in cap3 than in cap1 and cap2. Similarly, in cap3 traces of an unknown with *m/z* of 144.08 is found, but this unknown is produced at high relative abundances only in cap1 (Appendix A.6). These observations support the idea that structural genes are present in all ESUs, but ESUs differ in the extent to which they are down or upregulated.

Modifications in gene regulation may be a fast and simple mechanism for differential expression of metabolites between species. This would allow for rapid defense evolution and explain why close relatives are divergent in defenses. Major shifts in defenses would help to neutralize the advantages that short-lived herbivores have in an evolutionary arms race with long-lived trees.

Conclusions

The results from a number of recent studies suggest that herbivores play an important role in trait diversification and speciation in plants. Our functional trait approach provides evidence for enemy-related differentiation among closely related lineages. That marked phenotypic differences occur in defensive traits and not in other traits between sister lineages within a clade lead us to hypothesize that selection exerted by herbivores is one of the main ecological factors driving diversification. This interpretation is consistent with the proposal that the time scale for changes in abiotic selective pressures may be much longer than for natural selection due to biotic factors (Schemske 2002; Coley & Kursar 2014). Thus, plant traits that are adaptations to the arms race may evolve quickly in order to track counter-adaptations from their enemies. Simple and fast changes in defenses through gene regulation are consistent with this hypothesis.

Because four of the ESUs studied here co-occur as neighbors, our findings have significant implications for coexistence. In the tropics, growing evidence is showing dissimilarity in defenses between close relatives occurring in sympatry. Thus, divergent selection on defensive traits by herbivores might be mandatory for coexistence of closely related neighbors in tropical forests, and could potentially explain the astonishingly high local diversity of these forests (Coley & Kursar 2014).

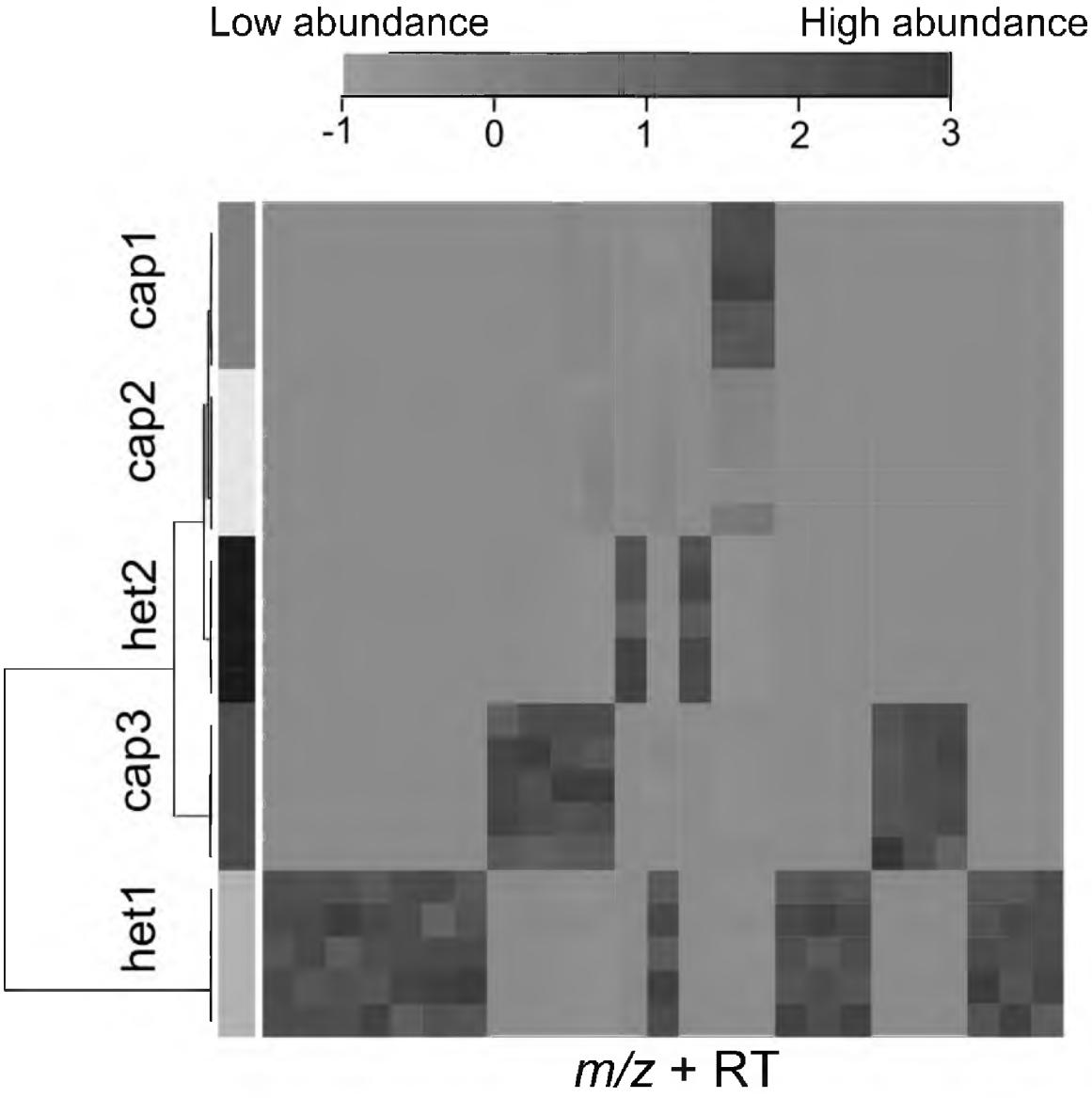


Figure 3.1. Heatmap of a hierarchical clustering of *I. capitata and I. heterophylla* ESUs based on relative abundances of the most important 25 UPLC-MS phenolic metabolites. Each column represents a metabolite with a unique *m/z* and retention time; analyses are based on 5 individuals per ESU. Each row is one UPLC-MS analysis from one individual plant. Metabolites were identified as "important" based on ANOVA analysis. The color scale for metabolite relative abundance is based on signal intensity (total ion current from the mass spectrometer).

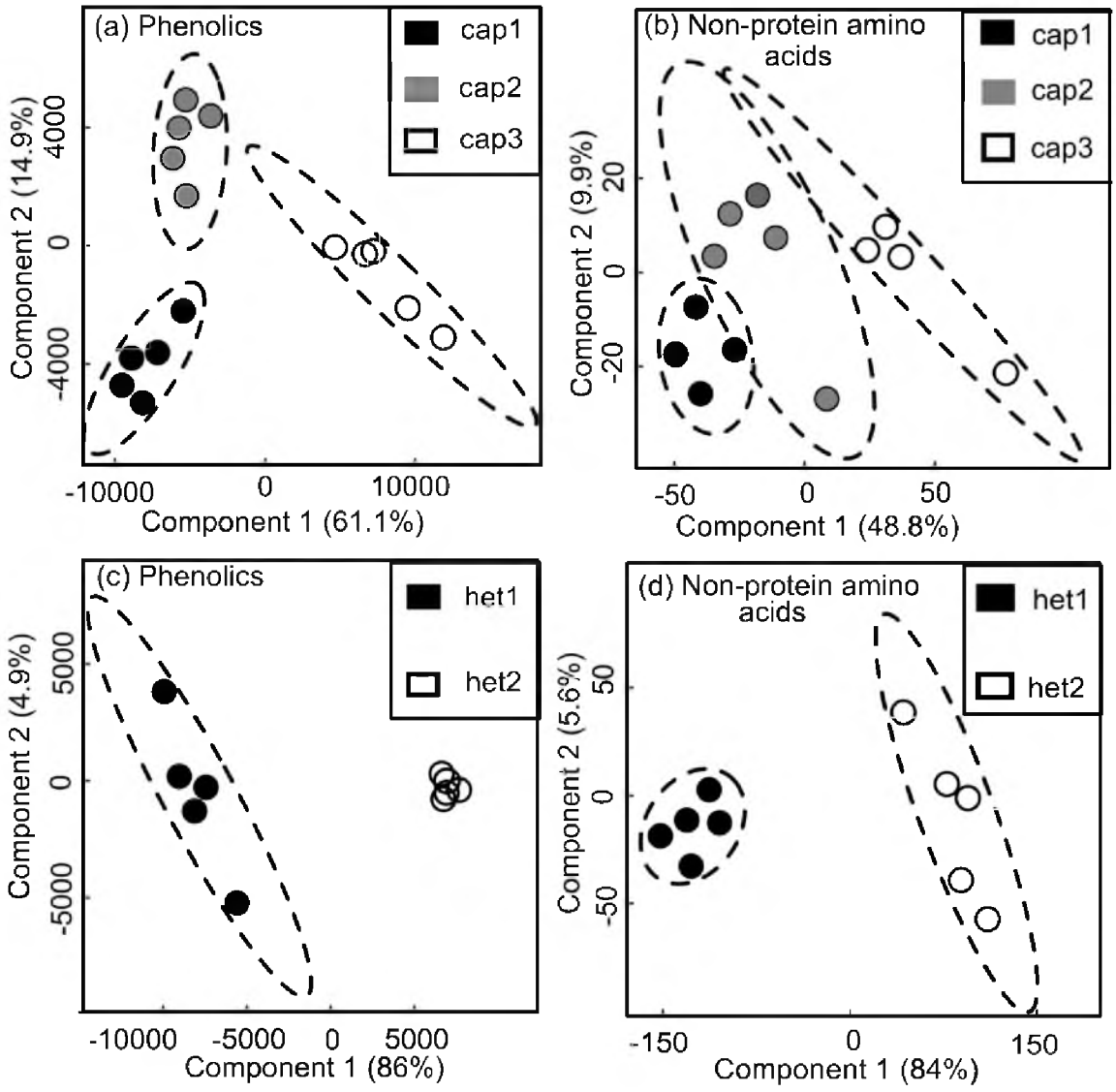


Figure 3.2. Score scatter plots from a PLS-DA model fitted to the relative abundances of peaks obtained by metabolic fingerprinting. (a) and (b) *I. capitata* species complex. (c) and (d) *I. heterophylla* species complex. The percentage of the variation explained by each component is indicated on the axes. The ellipses delimited by the dotted lines represent the 95% confidence regions. P values are provided in the Results.

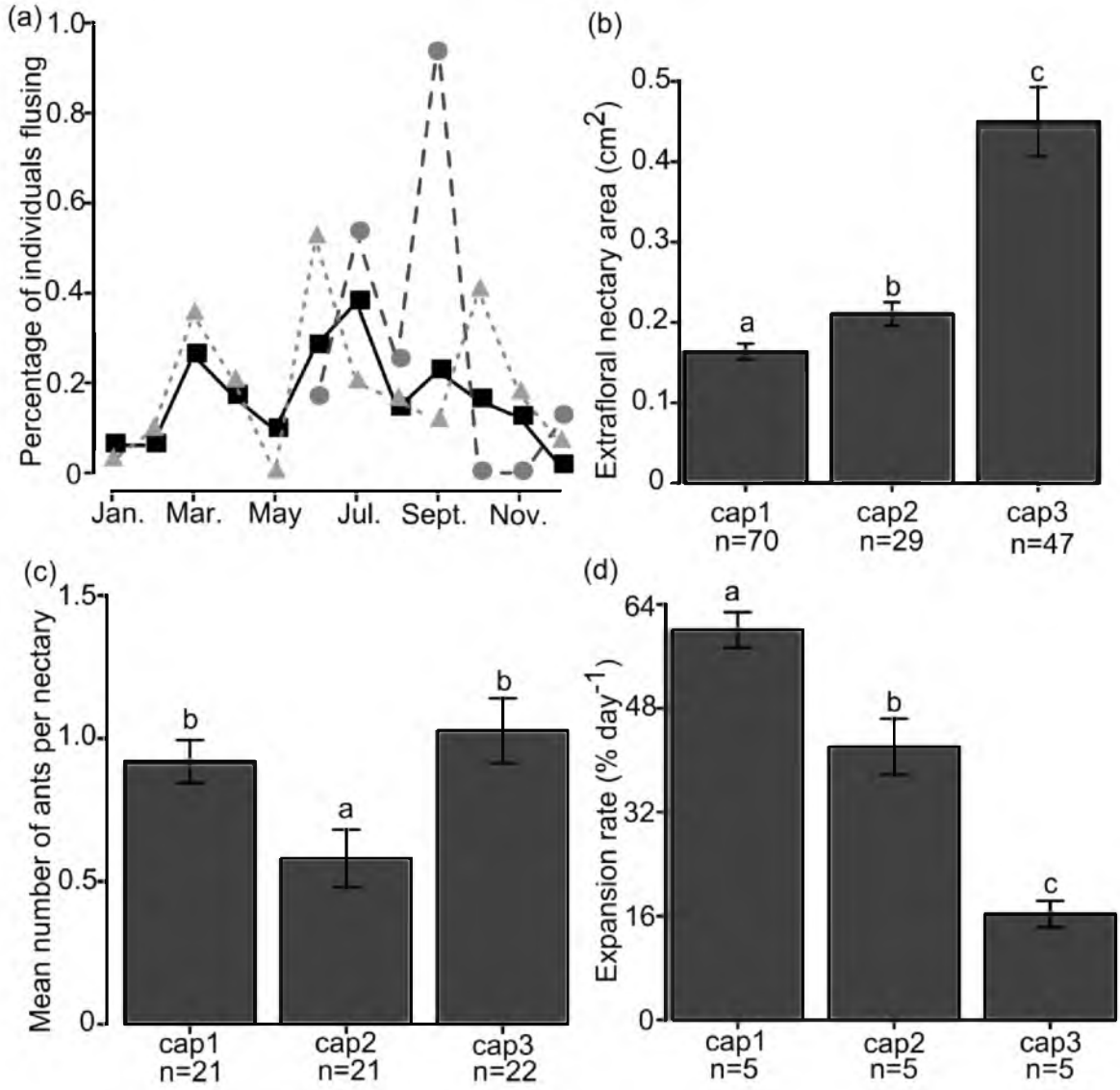


Figure 3.3. Non-chemical defensive traits of leaves for the ESUs of the *I. capitata* species complex. In panel (a) circles represent cap1 (n=24), squares represent cap2 (n=65), and triangles represent cap3 (n=74). Letters denote significant differences between ESUs. Bars are mean ± SE.

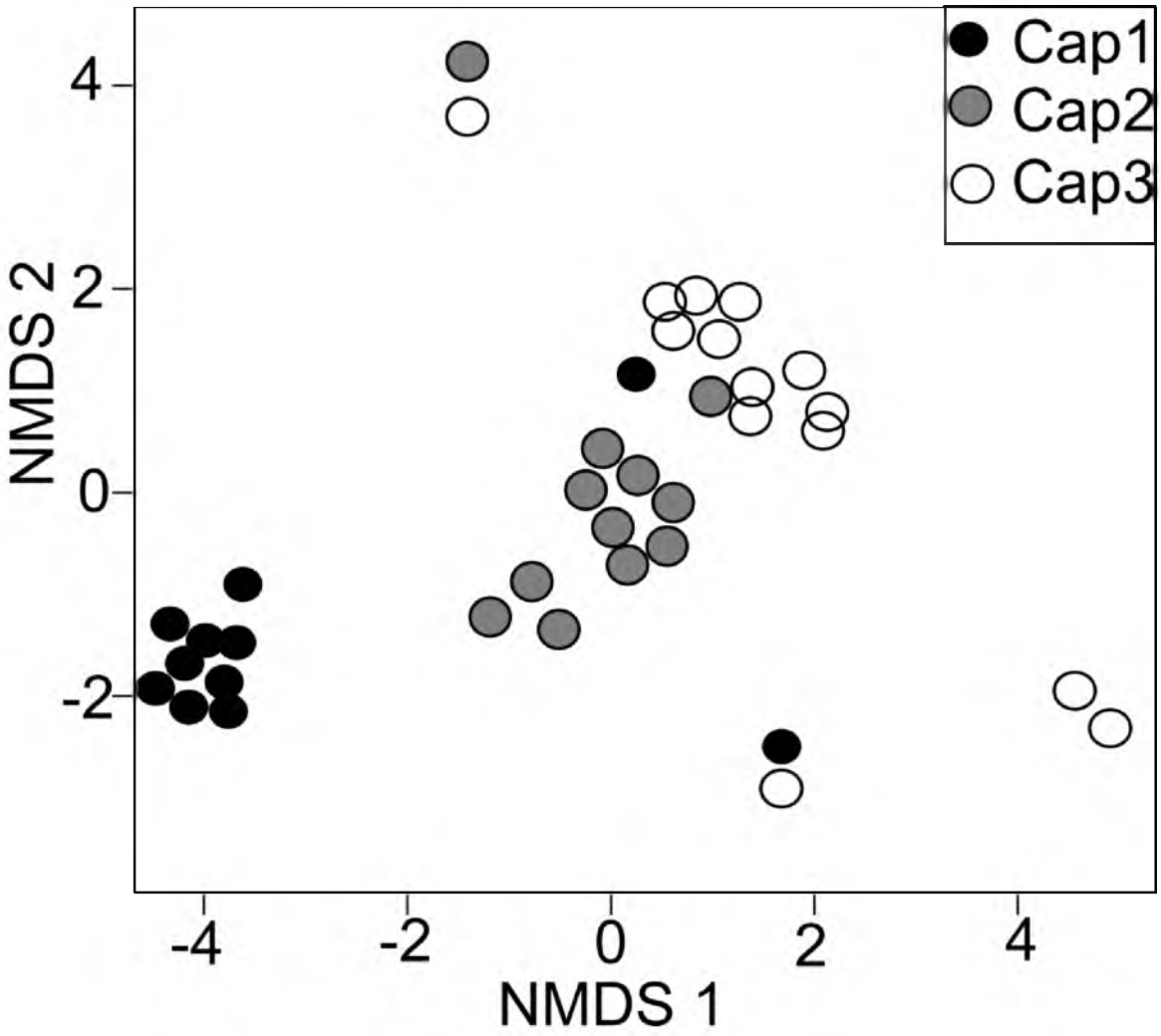


Figure 3.4. Ordination diagram of 38 *I. capitata* plants based on the similarities of their insect herbivore faunas (stress value= 0.05). Similarities in herbivore composition were calculated with the Bray-Curtis Index.

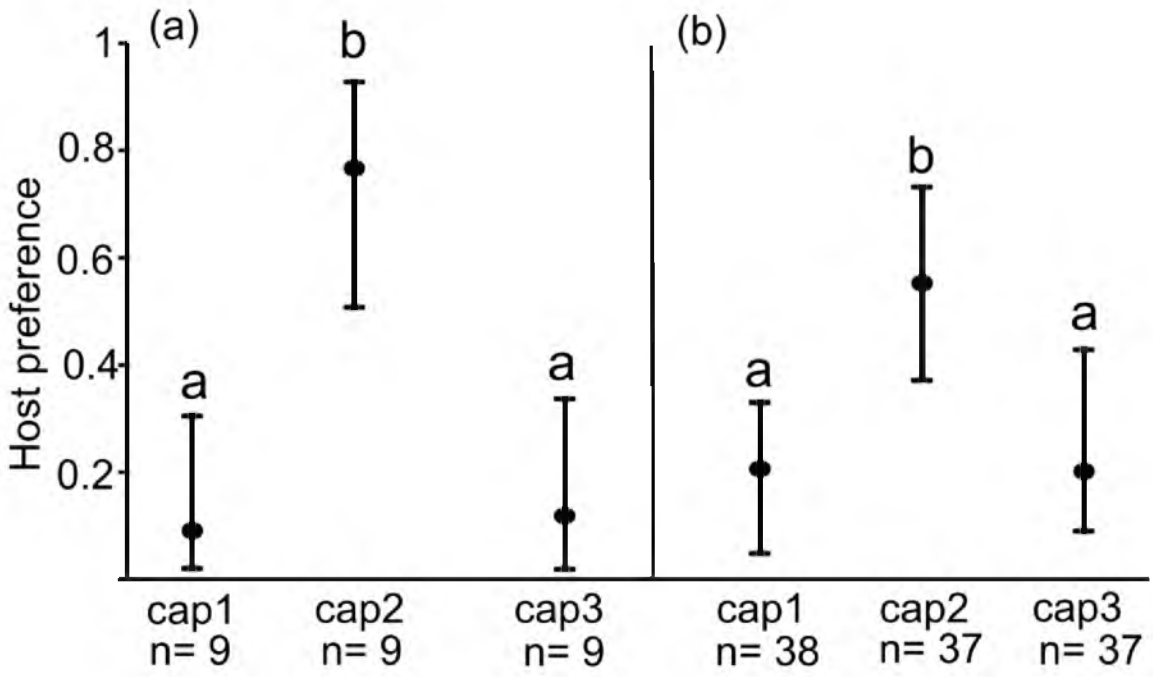
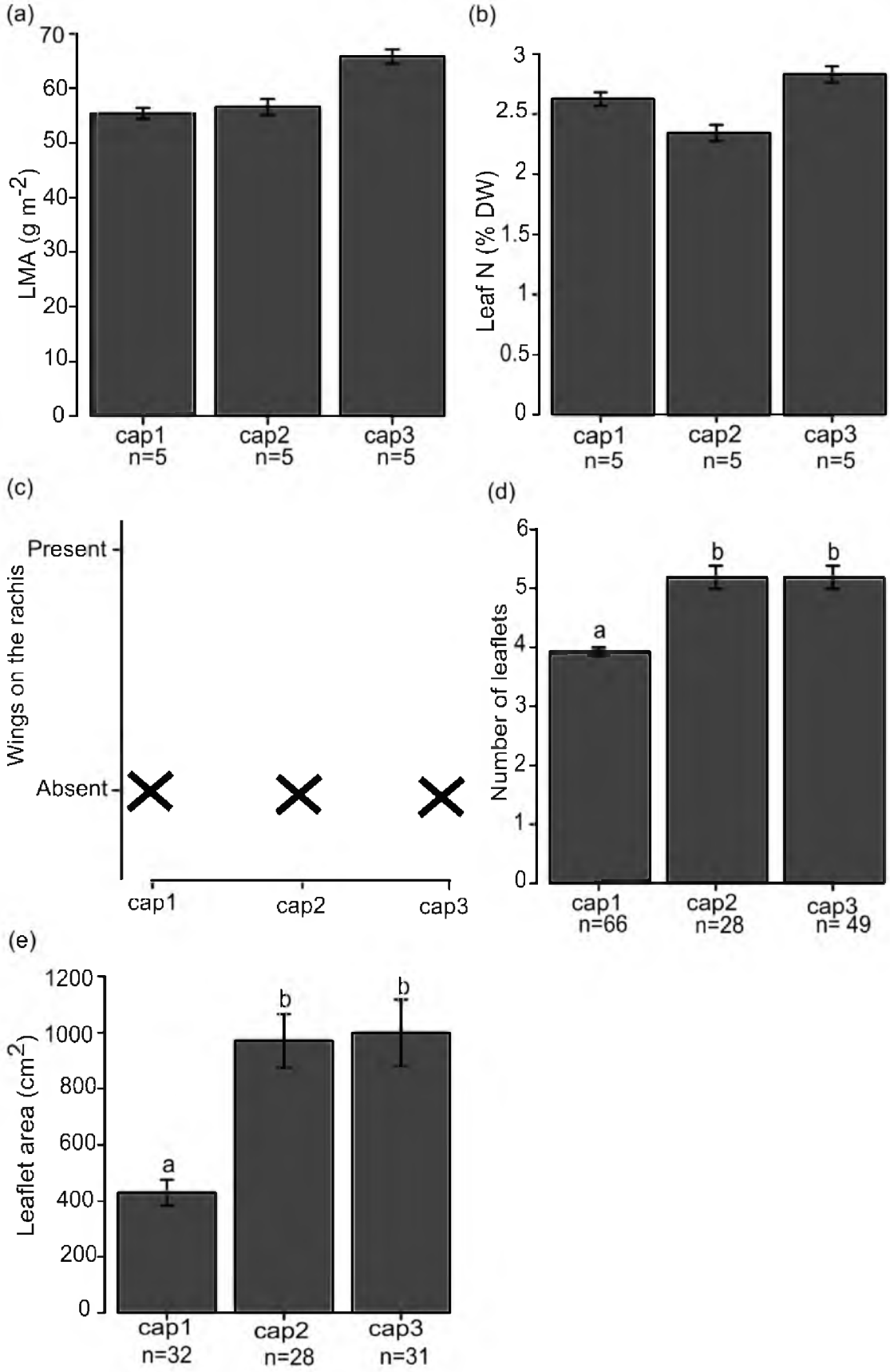


Figure 3.5. Summary of sawfly host preferences in: (a) choice experiment and, (b) field survey. In both cases, preference is plotted as the median and 95% confidence interval of the posterior probability distribution for population preference, estimated from a hierarchical Bayesian model (Fordyce *et al.* 2011). Lower-case letters denote posterior probabilities of >0.95 for differences in preferences.

Figure 3.6. Non-defensive traits of leaves for the ESUs of the *I. capitata* species complex. Letters denote significant differences between ESUs. Bars are mean ± SE. LMA=Leaf mass per area, N= Nitrogen.


(a)
LMA (g m-2)
70
60
50
40
30
20
10
0
cap1 n=5
cap2 n=5
cap3 n=5
(b)
Leaf N (% DW)
3
2.5
2
1.5
1
0.5
0
cap1 n=5
cap2 n=5
cap3 n=5
(c)
Wings on the rachis
Present
Absent
cap1
cap2
cap3
(d)
Number of leaflets
6
5
4
3
2
1
0
a
b
b
cap1 n=66
cap2 n=28
cap3 n= 49
(e)
Leaflet area (cm2)
1200
1000
800
600
400
200
0
a
b
b
cap1 n=32
cap2 n=28
cap3 n=31

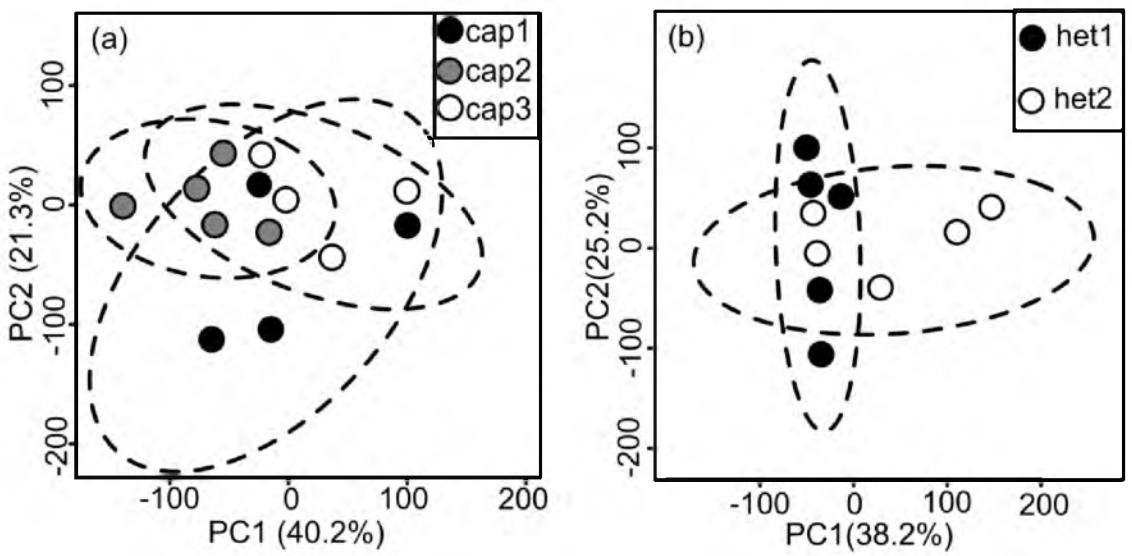


Figure 3.7. Score scatter plots from a PCA model fitted to GC-MS data of primary metabolites. (a) *I. capitata* species complex. (b) *I. heterophylla* species complex. The percentage of the variation explained by each component is indicated on the axes. The ellipses delimited by the dotted lines represent the 95% confidence regions.

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CHAPTER 4

TESTING THE COEVOLUTIONARY ARMS RACE: A CASE STUDY WITH THE GENUS OF TREES *INGA* AND ITS HERBIVORES

Abstract

The "arms race" paradigm predicts that interactions between plants and insects may drive diversification in both groups and has led to evolutionary conservatism in plant defenses and in host use by insect herbivores. We tested these expectations using the Neotropical genus of trees *Inga* (Fabaceae) and its associated lepidopteran herbivores in the Peruvian Amazon. We constructed multilocus phylogenies for both plants and insects and collected data on host associations and plant defensive traits to examine patterns of host use with respect to the host plant defensive traits and host plant phylogeny, and to determine the role of coevolutionary interactions in shaping herbivore host selection and defense divergence. Contrary to expectations, we found that host defensive traits that are important in host selection, such as secondary chemical compounds, recruitment of protective ants, leaf nutritional quality, physical defenses and food availability, show little phylogenetic conservatism. That is, closely related species of *Inga* are generally not more similar in defenses than are more distantly related *Inga*. We also found that *Inga* species pairs that are more similar in herbivore communities are also more similar in

defensive traits but not necessarily more closely related. Host defensive traits explained a substantial fraction of the variation (40%) of the herbivore community similarity, with secondary chemistry being the most important host defense. Analysis at finer taxonomic scales revealed that different families of lepidopteran herbivores select hosts based on different defensive traits. Although few studies characterize plant traits important for host selection, our results suggest that differences among families of herbivores in their physiologies, ecology and natural history are reflected in trait associations. Finally, we compared the phylogeny of *Inga,* the defenses of *Inga* and the phylogeny of the most abundant family of herbivores. The results suggest that closely related species of herbivores feed on species with similar defenses rather than on closely related plants, a pattern more consistent with ecological resource tracking than with the arms race model of coevolution or cocladogenesis. When taken together, these results show that, as expected, plant defenses determine host choice. But, they also strongly suggest that plant-antiherbivore defenses evolve rapidly and that herbivore traits involved in host choice evolve more slowly and depend more on existing host-choice traits. Hence, there is an apparent asymmetry in the interaction between *Inga* and its herbivores. Specifically, although divergence in herbivores might not be driven by their interactions with plants, herbivores may be an important factor driving the divergence among plant species.

Introduction

Fifty years ago, Ehrlich & Raven introduced a seminal concept that has dominated our understanding of the interactions between plants and their natural enemies. They proposed that the adaptations of butterflies and plants are reciprocal and their

interactions may have driven diversification in both groups (Ehrlich & Raven 1964). In this view, plant and insect associations are the product of strong coevolutionary histories. Nevertheless, it is unclear whether or not these groups have experienced reciprocal coevolution and cocladogenesis (Thompson 1994; Janz 2011). Many studies solely compare the congruence between insect and host-plant phylogeny with the expectation that more closely related hosts will share closely related herbivores. The alternative hypotheses, that plant defensive traits or herbivore adaptations evolve rapidly, are not tested. Such analysis would require not only phylogenetic histories for both plant and herbivores, but also data on both the traits of plants and herbivores. Here, we focus on including extensive characterizations of plant anti-herbivore traits as well as phylogenies for both groups of organisms to robustly examine the predictions from the coevolutionary theory.

One expectation arising from the Ehrlich & Raven's paradigm is phylogenetic conservatism in host defenses. In other words, closely related species of plants should have similar defenses. Because there are strong constraints on biosynthetic pathways for particular defenses (Liscombe *et al.* 2005), and the major mode of evolution for defenses is generally assumed to be through gene duplication (Ober 2010), similarity in chemical defenses among close relatives is expected. Although explicit tests are few, all have detected low phylogenetic signal for defenses (Becerra 1997; Agrawal & Fishbein 2006; Kursar *et al.* 2009; Sedio 2013). This has resulted in a pattern of poor congruence between their phylogenetic histories and the expression of anti-herbivore traits. These results call for alternative hypotheses. The fact that closely related species are dissimilar

in defenses suggests that these traits experience rapid evolutionary change and that herbivores are actually selecting for divergence in defensive traits.

Another expectation from the "arms race" hypothesis is that host usage is conserved in phytophagous insects at both ecological and evolutionary time scales. That is, because plant anti-herbivore traits are assumed to be phylogenetically conserved, then host shifts in insects are predicted to be rare, and, if shifts happen, these should be between closely related plants (Agrawal 2007; Futuyma & Agrawal 2009). Thus, at the ecological level, the assemblage of herbivores in a local community is expected to be related to the phylogenies of the assemblages of the host plant species (Lewinsonh *et al.* 2005; Odegaard *et al.* 2005; Weiblen *et al.* 2006). However, the phylogeny of the hosts has been found to be a poor predictor of herbivore assemblages (Novotny *et al.* 2010).

At an evolutionary level, parallel cladogenesis, or similarity in the sequence of speciation events between both groups is expected (Mitter & Brooks 1983). However, insects appear to shift hosts much more frequently than expected (Agosta 2006; Janz 2011), and host shifts among closely related herbivores are more strongly correlated with the chemistry of the new hosts than with their phylogenetic relationships (Becerra 1997; Becerra & Venable 1999; Berenbaum 2001; Wahlberg 2001). In addition, phylogenies of insects rarely match those of their hosts (Futuyma & Agrawal 2009; Thompson 1994). It appears that the most common pattern of evolutionary interactions is either by sequential evolution (Futuyma & Agrawal 2009) or by resource tracking (or host trait tracking, Agosta 2006; Brooks & McLennan 2009; Janz 2011). Thus, the wide acceptance of a strong phylogenetic signal for defenses in plants and, hence, for host use in herbivores may not be warranted, suggesting that these ideas need more rigorous testing.

Here, we present an assessment of plant-herbivore coevolutionary theory that combines phylogenies for both with an extensive evaluation of plant defensive traits. Our goal is to determine the role of coevolutionary interactions in shaping herbivore host selection and defense divergence. We focus our study on the species-rich Neotropical genus of trees *Inga* (Fabaceae) and its lepidopteran herbivores in the Peruvian Amazon. We characterized many classes of chemical compounds in leaves (Kursar *et al.* 2009), together with recruitment of protective ants, leaf nutritional quality, physical defenses and food availability. We also quantified the identity and abundance of Lepidoptera associated with species of *Inga,* and developed multilocus phylogenies for plants and for herbivores from the most abundant family, Elachistidae. Because previous studies indicated that chemical defenses evolve rapidly within *Inga* (Kursar *et al.* 2009), we predicted that more closely related plants would not have similar defenses, and that herbivore host choice in *Inga* will show phylogenetic conservatism with respect to host defenses but not host phylogeny; that is, closely related species of herbivores will choose plants with similar defenses rather than closely related hosts.

Methods

Study site

This study was carried out at the Los Amigos Research Center (12°34 S, 70°05 W; elevation ~270 m) located in the lowland Amazon region of Madre de Dios, Peru. Los Amigos comprises 453-ha of primary tropical rainforest on a mixture of upland terraces and floodplains. Annual rainfall at Los Amigos is between 2700-3000 mm, and the mean monthly temperature ranges from 21 - 26°C (Pitman 2008).

Study species

Inga is a canopy tree in the Fabaceae (mimosoid), and is found in lowland moist forests through the new world. There are over 300 described species (Pennington 1997). At Los Amigos and elsewhere, the genus *Inga* constitutes one of the most abundant and diverse genera (N. Pitman *unpublished data*), with more than 40 species occurring in 25 ha and 6% of the stems (Valencia *et al.* 2004). We collected data on 42 species of *Inga*. We focused our study on understory saplings, a key stage in the life cycle of a tree (Green *et al.* 2014).

Inga is associated with several groups of herbivores including Coleoptera, Orthoptera, phloem-feeding Coreidae, Diptera, sawflies, Phasmida and Lepidoptera. However, the group causing the most damage to leaves of *Inga* is Lepidoptera (Kursar *et al.* 2006). For this study we focused only on lepidopterans due to their importance.

To record host associations of lepidopteran herbivores, we visually searched c. 30 young leaf flushes per tree species and collected only those larvae that were found feeding. Insects were collected by hand from the leaves between 2010 and 2011 for a period of 10 months. All caterpillars were assigned to morphospecies in the field and subsequently to MOTUS (Molecular Operational Taxonomic Units) in the laboratory using sequences from the mitochondrial gene cytochrome oxidase I (*COI*, see below). We recorded a total of 1567 individuals in 174 MOTUS from 19 families of Lepidoptera (Appendix B.1).

Plant Defensive Traits

We focused our study on the defenses of expanding leaves because during this ephemeral stage they receive more than 75% of the damage accrued during the lifetime of a leaf (Coley & Aide 1991; Kursar & Coley 2003; Brenes-Arguedas *et al.* 2008). Therefore, the defensive traits most relevant for insect herbivores when selecting hosts would be those of young leaves.

We measured multiple defensive traits that capture the entire plant's defensive profile. We recorded the presence or absence of several classes of phenolic compounds, saponins, and metabolites containing primary or secondary amines that have been shown to decrease the growth and survival of herbivores (Lokvam *et al.* 2006; Brenes-Arguedas *et al.* 2008). Details on chemical procedures are reported in Kursar *et al.* 2009.

We assessed the length and density of trichomes per area (number of hairs per 2 cm^{-2}) in c. 30 individuals per species. Young leaves are also defended against herbivory by expanding leaves rapidly and delaying the development of the chloroplast (Kursar & Coley 1992a). Leaf expansion rate was determined as the percent increase in area per day for c. 13 individuals per species. Chloroplast development was measured as the chlorophyll content (mg dm^{-2}) of leaves between 30% and 80% of full expansion for c. 30 individuals per species.

The timing and synchrony in leaf production are two measures of food availability for insect herbivores (Aide 1993; Kursar & Coley 2003). To measure these traits, we monitored between 30 and 70 individuals per tree species for monthly leaf production. To estimate timing in leaf production we calculated the *mean angle* (using circular statistics), which indicates the average date of peak flushing activity across all individuals (Zar

1999). To estimate synchrony in leaf production we calculated the coefficient of variation (cv) of the number of plant individuals per species flushing each month.

Inga leaves have extra-floral nectaries that produce nectar and attract protective ants only during the short period of leaf expansion. We determined the identity and the abundance of ants visiting these nectaries (# of ants per nectary) in c. 30 individuals per species. See Appendix B.2 for detailed methods.

Herbivore Phylogenies

Phylogenetic analyses for the most abundant lepidopteran family, Elachistidae (Appendix B.1) were inferred using 1-3 individuals per MOTU (for MOTU assignment see below) and three gene fragments: nuclear Elongation-factor (*EF-1α*) and wingless (*Wg*), and mitochondrial *COI*. For DNA extraction, legs, but sometimes larger body parts of larvae were used. The remaining parts were preserved to be used as vouchers. We extracted total genomic DNA from single body parts in 50 ul of extraction buffer containing 5% Chelex 100 resin (Bio-Rad, Hercules, CA) as described in West *et al.* (1998). For *COI*, PCR amplification and DNA sequencing for most part of our samples were generated at the Canadian Center for Barcoding using standard barcoding protocols (Ivanova 2006, deWaard 2008). For the nuclear gene fragments and the remaining samples for *COI* we performed PCR amplification with 1 ul of DNA extract, 0.2 μM of each primer, and 10 ul of Multiplex PCR kit (Qiagen) in a 20 ul reaction volume (Appendix B.3). We used the same pair of primers for both amplification and sequencing. For *COI* we used the primers LepF1 (5' ATT CAA CCA ATC ATA AAG ATA TTG G 3') and LepR1 (5' TAA ACT TCT GGA TGT CCA AAA AAT CA 3') (Hebert *et al.*

2004), amplifying a 658 bp coding region. For *EF-1α* we used the primers EF51.9 (5' CAR GAC GTA TAC AAA ATC GG 3'), and EFrcM4 (5' ACA GCV ACK GTY TGY CTC ATR TC 3') (Cho et al. 1995), amplifying a region of 511 bp. For *Wg* we used two sets of primers. Initially, we used the primers LepWg1 (5' GAR TGY AAR TGY CAY GGY ATG TCT GG 3') and LepWg2 (5' ACT ICG CAR CAC CAR TGG AAT GTR CA 3') (Brower and DeSalle 1998), amplifying a region of 403 bp. Because of difficulties with these primers in some MOTUS, we designed specific internal primers, LepWg1_mod (5' GAA TGT AAR TGT CAY GGY ATG TCY GG 3') and LepWg2_mod (5' GAT ACC CTC KIC CRC ARC AC 3') based on sequences already obtained. For all taxa, we also tried to amplify Phosphoenolpyruvate carboxykinase (*PEPCK*), long wave-length opsin (*opsin*) and the carbamoyl-phosphate synthetase domain of (*CAD*) protein, but due to low success rate we omitted these genes from the analyses. PCR products were purified using a shrimp alkaline phosphatase protocol. Sequencing was performed using ABI BigDye chemistry (Perkin Elmer Biosystems, Waltham, MA) on ABI 3730xl capillary sequencer. We sequenced all products in both directions. The sequences were assembled into contigs and manually edited using the program Sequencher v 5.1 (Gene Codes). The resulting sequences were subsequently aligned using the program MUSCLE (Edgar 2004).

For MOTU assignment we used COI sequence divergence. Aligned sequences were clustered into MOTUS by using the software package jMOTU (Jones *et al.* 2011), with a similarity cutoff of 15 bp (Appendix B.4). These results were compared with the MOTUS generated by the Automatic Barcode Gap Discovery method (ABGD, Puillandre *et al.* 2011). Both methods rendered identical results. Taxonomic annotation up to the

family level was added to each MOTU by BLASTing each consensus sequence against the NCBI BLAST web interface.

Phylogenetic relationships for Elachistidae were inferred using multilocus coalescent-based Bayesian species tree in *BEAST 2.2.0 (Heled & Drummond 2010), with substitution models and codon partition for each marker according to the suggestions of PartitionFinder 1.1.0 (Lanfear *et al.* 2012). The models employed for the partition analyses were: TN93 for COI and HKY for EF1α and *Wg*. A strict clock with a constant population size and a Yule speciation process as tree priors were used. Alternative models were assessed using Bayes factors (BF, Kass & Raftery 1995). Parameters were estimated from 3 independent runs of 100 million generations combined using LogCombiner 1.8 (Drummond & Rambaut 2007) with a burn-in of 10 million generations and sampling every 10,000 generations in each run. BEAST model convergence was confirmed by examination of parameter estimate distribution in Tracer 1.6.

Plant Phylogeny

Phylogenetic relationships between *Inga* host species were inferred using seven chloroplast regions (rpoCI, psbA-trnH, rps16, trnL-F, trnD-T, ndhF-rpl32, rpl32-trnL) and the nuclear ribosomal internal transcribed spacer regions (ITS). DNA extractions used a modified CTAB protocol (Doyle & Doyle 1990) or DNAeasy plant mini kits (Qiagen). PCR and sequencing protocols for chloroplast regions are given by Kursar *et al.* (2009) and for ITS by Richardson *et al.* (2001) and Dexter *et al.* (2010). Sequences were assembled using Sequencher v4.5 (Gene Codes Corp., Ann Arbor) and aligned

manually, which was unproblematic given low sequence divergence. Sequences aligned using MAFFT v7.0 (Katoh & Standley 2013) and phylogenies estimated using a maximum likelihood framework using RAxML with separate models for ITS and cpDNA (Stamatakis 2006). Phylogenies were subsequently time-calibrated using penalized likelihood (Sanderson 2002), where the crown age was constrained to 6 myrs (following Richardson *et al.* 2001; Lavin 2006).

From the resulting tree, we extracted pairwise distances between *Inga* species using the function 'cophenetic' in the APE package (Paradis *et al.* 2015) from the statistical programming language R version 3.1.2 (R Development Core Team 2014). This phylogenetic distance matrix was used in all the subsequent ecological analyses that involved the phylogeny of *Inga.*

Statistical Analyses

Relationship between plant traits and phylogenetic signal

Associations between defense traits were investigated by using matrix correlation analyses and phylogenetically independent contrasts (PICs, Felsenstein 1985). Correlations between continuous defense traits were analyzed using linear models with PICs forcing the intercept through zero (Garland *et al.* 1992) in the APE package. To estimate the relationship between continuous and non-continuous traits (e.g., of non-continuous traits: presence or absence of chemical compounds, ant visitor community to extra-floral nectaries and the timing of leaf production), we calculated the distances between pairs of *Inga* species for each trait and examined their correlations using partial Mantel tests, conditioned on a matrix of phylogenetic distances between *Inga* species.

The distance matrices for each trait were standardized by the maximum observed distance and calculated using the Manhattan dissimilarity index. Because the timing in leaf production is a circular variable (*mean angle*), we used the angular separation method from the package circular (Lund & Agostinelli 2015) to calculate the distance matrix for this trait.

We also performed a PCA on continuous trait data to derive independent axes of defense variation. For the significant axes (axes that showed eingenvalues >0.7, Jolliffe cut-off, Jolliffe 1986), we evaluated the phylogenetic signal by using the analyses of Bloomberg's $\bar{K}$ (Bloomberg *et al.* 2003). If there is no phylogenetic signal, $\bar{K}$ would be close to zero, whereas values approaching one would indicate that the trait value matches expectations under a Brownian model of evolution. For non-continuous traits, we examined the phylogenetic signal by estimating the correlation between the distance matrix for each trait and the phylogenetic distance between pairs of *Inga* species using Mantel tests.

Constraints on host plant selection

To examine if differences in total herbivore community structure were related to differences in phylogenetic relationships and/or defensive traits between pairs of *Inga* hosts we used Mantel and partial Mantel tests with 9999 permutations. All the feeding records that were limited to a single individual in a particular host were not included in these analyses. Overlap in feeding records among hosts was estimated using the Bray-Curtis dissimilarity index with standardized abundance raw data. The resulting matrix was then compared with a phylogenetic distance matrix for *Inga* hosts and with a defense

distance matrix conditioned on the phylogenetic pairwise distances between species. The defense distance matrix between *Inga* species was generated by averaging the distance matrices for the different defense traits such that they were all weighted equally. These analyses were performed in the vegan package (Oksanen *et al.* 2015).

To quantify the extent to which host phylogeny and/or host defenses structure herbivore community and to determine which defense trait is more important, we used Redundancy analyses (RDA). The herbivore community similarity matrix was used as a response variable in a partial dbRDA (distance-based Redundancy Analysis) together with each one of the measured defensive traits and the principal coordinates of the phylogenetic distance matrix as explanatory variables. This analysis was performed without timing and synchrony in leaf production because we were lacking data on these traits for some species. The analyses were run using sampling effort as a covariable. First, we ran a global test using all the explanatory variables and because this analysis was statistically significant ($p \leq 0.05$, 9999 permutations), we performed variable selection using the function ordistep. We did this to avoid overfitting and to select the most parsimonious model. Adjusted R^2 values for the selected model were computed using the function varpart. We also ran a more restricted analysis that included only species for which we had data on phenology of leaf production. No results were affected (Appendix B.5). We performed these analyses in the vegan package.

Phylogenetic patterns of host use

To examine if different groups of herbivores have diversified responses to plant traits and phylogeny, we used two different approaches. (1) We performed the above

analyses for each one of the most abundant families (Appendix B.1: Elachistidae, Erebidae and Riodinidae), where the overlap in herbivore communities between *Inga* species was estimated with the Bray-Curtis index; and (2) We ran analyses that included joint-absence information. For this, we constructed a series of maximum likelihood models. Each family was modeled separately. We modeled the probability of occurrence (p) using a binomial distribution with the number of trials equals to the total number of herbivore species found for each *Inga* species. Models that incorporated the effects of one or more defensive traits and the principal coordinates of the phylogenetic distance matrix were fit to these data in the packages bbmle (Bolker 2015a) and emdbook (Bolker 2015b). The models were run using sampling effort as a covariable. We performed model comparison based on Akaike's Information Criterion for small sample sizes (AICc).

To investigate if past host shifts have occurred more often on *Inga* that are more similar in defenses or on *Inga* that are more closely related, we examined the congruence of the Elachistidae phylogeny with *Inga* phylogeny and *Inga* defenses using ParaFit (Legendre *et al.* 2002) from the APE package. This statistical tool tests the significance of a hypothesis of coevolution between parasites and hosts using phylogenetic distance matrices of associated taxa and a set of host-parasite links. Distance matrices for herbivores and plants were derived from their phylogenetic trees and from a bio-neighbor joining tree we obtained from the total defense distance matrix using the 'cophenetic' function in the APE package. We ran this analysis with 9999 permutations.

Results

Are leaf defense traits correlated and orthogonal?

Of the eight anti-herbivore traits measured, three pairs of these covaried (Table 4.1). For example, species with a higher density of leaf trichomes also exhibited longer trichomes ($\bar{r}^2$=0.79, p<0.001). Species with higher leaf expansion rates exhibited lower leaf chlorophyll content ($\bar{r}^2$=-0.55, p< 0.001). And, species that were more similar in the mean number of ants visiting the extra-floral nectaries, were also visited by similar species of ants (Mantel *r*=0.28, p= 0.02). Other traits were uncorrelated.

Consistent with the patterns of trait correlation, analysis for orthogonality on the six continuous traits revealed four independent defense mechanisms (physical, developmental, biotic defenses and food availability, Table 4.2). Specifically, PCA analysis determined four significant components of defense variation (axes that showed eigenvalues >0.7, Jolliffe cut-off, Jolliffe 1986), with each axis highly correlated with a different defense mechanism. The first axis was highly correlated with trichome density and length (physical defenses, r= 0.58 and r=0.6, respectively), the second axis with leaf expansion rate and chlorophyll content (developmental defenses, r=0.60 and r=−0.69, respectively), the third axis with the mean number of ants visiting the extra-floral nectaries (biotic defenses, r=0.85) and the last axis with synchrony in leaf production (CV, a measure of food availability, r=−0.70).

Are closely related *Inga* similar in defenses?

In general, species of the genus *Inga* in Los Amigos showed large differences in defenses among close relatives (Figure 4.1). For continuous traits, only physical defenses

(first PCA component) showed a phylogenetic signal ($\bar{K}$= 0.46, p= 0.01). However, the estimate of $\bar{K}$ was substantially lower than the expected value of 1 under Brownian motion evolution. For the non-continuous traits, only chemistry showed a weak pattern of phylogenetic conservatism (Mantel *r*= 0.17, p=0.02). We found no indications of significant phylogenetic conservatism for the other defense mechanisms.

Do defensive traits and/or phylogenetic relationships in *Inga* correlate with host use by herbivores?

Results from the matrix correlation analyses revealed that overlap among *Inga* hosts in their full assemblage of lepidopteran herbivores gradually decreases with an increase in defense distance between *Inga* hosts (Partial Mantel *r*= 0.5, p= 0.01, Figure 4.2a, Table 4.3). In other words, pairs of *Inga* species that are more similar in their defensive profiles are more likely to be fed on by a similar suite of herbivores. The same pattern was observed for phylogenetic distances between *Inga* hosts, although the relationship was considerably weaker (Partial Mantel *r*= 0.25, p=0.02, Figure 4.2b, Table 4.3).

Plant defensive traits explained much of the variation in lepidopteran communities feeding on different *Inga.* Distance-based RDA analyses at the herbivore community level identified host chemistry as the most important trait by explaining 30% of the total variation (chemistry R^2_{adj}=0.31, p=0.001). The ordination diagram supported this finding by clustering *Inga* species mainly by the expression of secondary metabolites (Figure 4.3), and to a lesser degree by physical defenses (trichome density and length R^2_{adj}=0.06, p=0.02).

Are there phylogenetic constraints on host selection?

The three most common families of herbivores showed quite distinct responses to host traits (Tables 4.3 & 4.4). For all families, similarity in herbivore assemblage consistently decreased with defense distance between the *Inga* plants (Table 4.3). In contrast, only Elachistidae showed a significant decline in community similarity with increasing phylogenetic distance between hosts (Table 4.3). Host choice by Riodinidae and Erebidae was not explained by plant phylogeny (Table 4.3).

For each family of herbivores, we investigated the specific role of each defensive trait in partitioning the variation in its herbivore community. We also modeled the response of each family with joint-absence information (Tables 4.4 & 4.5). Joint-absence means that the analyses include observations where herbivores were never collected on certain species of *Inga*. These analyses showed that the host-choice mechanisms in each clade of herbivores are divergent. Although secondary chemistry was consistently identified as an important variable, different families selected hosts based on different defensive traits. For example, phylogenetic relationships between *Inga* hosts was a robust predictor, again, only for the family Elachistidae. The phenology of leaf production was important to Erebidae, such that the species of *Inga* with flushing peaks in the months of March and October-November were preferred over species that flushed during other times of the year. Biotic defenses, such as the mean number of ants visiting the extra-floral nectaries, had a strong positive effect on the probability of occurrence of herbivores from the Riodinidae, a family in which caterpillars are tended by ants (Tables 4.4 & 4.5). Thus, more closely related herbivores feed on suites of plants with similar defenses, but herbivore clades are divergent in terms of which defenses matter most for host choice.

Phylogenetic patterns of host use

Parafit tests revealed that the evolution of Elachistidae and their *Inga* hosts has been independent ($p=0.5$, Figure 4.4a). In other words, there was no phylogenetic association between herbivores and plants; hence, more closely related Elachistidae do not feed on more closely related plants. In contrast, the herbivore phylogeny was not randomly associated with a dendrogram of the defenses of species of *Inga* ($p=0.05$, Figure 4.4b); that is, closely related species of Elachistidae fed on hosts with similar defenses.

Discussion

The classical theory of plant-insect associations predicts evolutionary conservatism in defenses among plants and in host specificity among insect herbivores. However, our data suggest that this is not necessarily the case. We found high divergence in defenses among close relatives of *Inga* (Figure 4.1), and that herbivore community structure in Lepidoptera correlates better with host defenses than with host phylogeny (Figure 4.2). Furthermore, host choice for the most abundant family of herbivores, Elachistidae, shows phylogenetic conservatism with respect to host defenses, but not host phylogeny (Figure 4.4). These results strongly suggest that herbivores might be an important factor in the evolution of plant defenses, and that plant defenses might be evolving relatively rapidly. It also implies that plant defenses determine the extent of host choice in both ecological and evolutionary time scales. If so, the fact that closely related Elachistidae use hosts with similar anti-herbivore traits suggests that herbivores may evolve slowly relative to plants. Hence, coevolution may be decidedly asymmetrical. We

suggest that improving our understanding of the ecology and evolution of plant-herbivore interactions will require close attention to plant defenses.

Anti-herbivore traits vary independently

Our analyses reveal that each class of defense has diverged considerably, even within a single genus. Anti-herbivore traits clearly fall into five independent axes of defense (Table 4.1): physical (trichome density and length), developmental (leaf expansion rate and chlorophyll content), chemical (secondary metabolites), biotic (ants visiting extra-floral nectaries) and phenological (synchrony and timing in leaf production). The covariance between some defenses, such as the negative relationship between chlorophyll content and leaf expansion rate, may result from functional trade-offs (Kursar & Coley 1992a; b & c), or to maximize defense (e.g., trichome number and length). But perhaps, most intriguing is that, to a large extent, defense categories are independent of each other (or orthogonal, Table 4.2), and also show substantial variation across species (Kursar *et al.* 2009). This result supports the hypothesis that the anti-herbivore defenses of plants provide a high-dimensionality niche space that may be important for understanding coexistence of plant species in tropical ecosystems.

Closely related species of *Inga* are highly divergent in defenses

A dominant paradigm in coevolutionary theory is that phylogeny reflects similarity in defenses among plants. Our comparative analyses found little relationship with phylogeny for physical defenses and secondary metabolites, and no evidence of phylogenetic signal for the other defense mechanisms, suggesting that close relatives tend

to be dissimilar in defenses (Figure 4.1). This lack of association between phylogenetic history and defense expression has also been found in *Asclepias* (Agrawal & Fishbein 2006), and in the Neotropical genera *Bursera* (Becerra 1997) and *Psychotria* (Sedio 2013). On the other hand, traits associated with habitat use and resource acquisition appear to be phylogenetically conserved within *Inga* (Endara *et al.* 2015) and *Psychotria* (Sedio 2013). This suggests that plant defenses are evolutionary labile and that rapid evolutionary change has occurred in response to herbivores.

Plant defenses are more important than plant relatedness for host use in herbivore community assembly

Consistent with the low conservatism of defensive traits in *Inga*, we found a weak relationship between overlap in the whole herbivore community and host phylogenetic distance (Figure 4.2b). Furthermore, this relationship was not consistent across all families of herbivores (Tables 4.3, 4.4 & 4.5). Plant relatedness had a significant effect only on the family Elachistidae, although it only explained a 14% of the total variation in the herbivore community (Table 4.4). Similarly, weak effects of host plant phylogeny on herbivore assemblages have also been documented in other communities (Ødegaard *et al.* 2005; Weiblen *et al.* 2006; Novotny *et al.* 2002; Novotny *et al.* 2010; Sedio 2013; Nakadai *et al.* 2014).

In contrast, similarity in defensive traits between *Inga* species seems to strongly constrain host selection at the whole-community level and across different families of herbivores (Tables 4.3 & 4.4, Figures 4.2a & 4.3). Remarkably, 30% of the variation in the whole herbivore community similarity was explained solely by chemistry, and it was

consistently identified as an important variable across insect families (Table 4.4). In other words, specific classes of secondary compounds matter (Figure 4.3). Certainly, secondary plant chemistry has been invoked as a major axis in host selection (Thompson 1988), and our results are consistent with this view. This is of particular interest because we considered a range of chemical defenses, including saponins, simple phenolics, flavonoids, tannins and diverse amines.

Despite the overall significance of chemistry on host selection, our analyses also revealed that different families of herbivores select hosts based on different defensive traits (Tables 4.4 & 4.5). Although very little has been published on the plant traits that determine host selection in tropical Lepidoptera, our analyses illustrate that the differences among families of herbivores in their physiologies, ecology and natural history is reflected in trait associations. For example, Elachistidae, the most abundant group of herbivores, is a family in which many of the species feeding on the expanding leaves of *Inga* had a leaf-mining habit. Leaf miners are known to show the highest level of host specialization among all the feeding guilds (Novotny *et al.* 2010; Forister *et al.* 2015), because of the intimacy of their association with the leaf. Therefore, it is not surprising that plant secondary metabolites and to a lesser degree phylogenetic relationships between *Inga* hosts were selected as significant predictors for Elachistidae occurrence (Tables 4.4 & 4.5). Similarly, chlorophyll content and the rate of leaf expansion, developmental defenses related to the time frame that leaves are tender and nutritious, were also selected as important variables for Elachistidae. Specifically, Elachistidae were more common on species of *Inga* with a relatively low rate of leaf expansion. Variation in leaf development could affect leaf miner survival, particularly for

species that require longer periods of time for successful development and are confined to a single leaf during their entire larval stage.

In contrast, for Riodinidae, ant associations with *Inga* (biotic defenses) explained as much as 30% of the total variation in their community similarity. For every unit of increase in the mean number of ants, the odds of occurrence for Riodinidae species in *Inga* increased by 22 times (proportional odds estimate for ants= 22.14, 95% CI (221.3 to 2.73)). Given that larvae of many species in this family are myrmecophytes (Fiedler 1991; Pierce *et al.* 2002), a strong effect of ants on host selection is expected. Myrmecophylous riodinids are tended by ants in exchange for food secreted by the larvae that contains carbohydrates and amino acids (Pierce & Mead 1981; Pierce 1985). Therefore, it has been suggested that ant-tended riodinids have been selected to feed upon protein-rich plants in order to meet the energetic requirements of their own development and the production of amino acids for the attendant ants (Pierce 1985). Our results are consistent with this hypothesis. Besides selecting ants as a strong predictor of Riodinidae occurrence, the model that included joint-absence information also selected leaf expansion rate as an important variable (Table 4.5), a trait positively correlated with the nitrogen content of expanding leaves (Kursar & Coley 2003). By contrast, for Lycaenidae, a sister family of Riodinidae, ants associated with *Inga* did not represent a significant factor for host selection (data not shown). This pattern was expected given that during our sampling, the species of Lycaenidae feeding on *Inga* were never found to be tended by ants. This result highlights the fact that, even for sister clades of herbivores, differences in plant defensive traits matter for host use.

With respect to Erebidae, much of the information available about their natural history is based on studies on a handful of temperate species of the subfamily Arctiidae ("tiger moths"). Studies on this group of herbivores indicate that, in general, they feed on several different hosts, but clearly prefer hosts that express certain classes of chemical compounds (Singer & Bernays 2009). Members of this group of herbivores are known for the opportunistic sequestration of chemicals from the host plant (Singer & Bernays 2009). In our sampling, almost 50% of the 38 MOTUS within Erebidae were members of the subfamily Arctiidae. The models with and without joint-absence information agreed in that leaf secondary chemistry and phenology (timing and synchrony in leaf production), were the most important variables for host selection by Erebidae (Tables 4.4 & 4.5). Thus, it might be that Erebidae feed on a restricted set of chemotypes (and host species) and that each host produces leaves at a different time of the year in synchronous, staggered episodes.

Host selection in Elachistidae shows phylogenetic conservatism with respect to host defenses but not to host phylogeny

Results at the macroevolutionary level are consistent with the strong constraints that host defensive traits appeared to exert on herbivore community assembly. For Elachistidae, we found no indication of host switching with respect to *Inga* phylogeny (Figure 4.4a). There was, however, a macroevolutionary pattern associated with the tracking of *Inga* defenses. Thus, more closely related Elachistidae clearly preferred species of *Inga* with similar defenses (Figure 4.4b), rather than closely related *Ingas*. Previous phylogenetic studies on host choices have revealed similar results. Apparently,

the evolution of host use in herbivorous insects seems to be relatively more conserved with respect to host defenses rather than to host phylogeny, not only at family level (Berenbaum 2001; Wahlberg 2001), but also at finer taxonomic scales (Becerra 1997; Becerra & Venable 1999). These results imply that herbivores are not tracking species per se but are tracking resources for which they have appropriate pre-adaptations for host finding, predator avoidance, and detoxification of plant chemicals (Agosta & Klemens 2008). Switches to novel hosts with divergent defenses would require simultaneous changes in many of these herbivore adaptations, and genetic variation for correlated innovations in a suite of traits is very unlikely (Brooks & McLennan 2002). Thus, it appears that closely related herbivores are similar in this complex set of adaptations, and consequently, constrained to feed on hosts with similar defenses.

Inga and its herbivores: a coevolutionary arms race?

The simplest model for the interactions among plants and their herbivores, which suggests reciprocal coevolution, does not adequately explain our results. Contrary to a scenario of defense and counter-defense as posited by Ehrlich & Raven (1964), there is an apparent asymmetry in the interaction between *Inga* and its herbivores. Our results provide strong support for the conclusion that *Inga* species are being selected by herbivores to diverge in their expression of defenses and that this leads to rapid divergence between closely related species of *Inga*.

In contrast, the selective regime for herbivores is less clear. We hypothesize that herbivores select hosts for which they are preadapted. This suggests that divergence in the traits of herbivore species might not be driven by their interactions with their *Inga*

host plants and that closely related species of herbivores may diverge slowly in the traits that determine host choice.

A key factor driving this asymmetry may be that plants and herbivores are members of a community, and, as such, they interact with a diverse assemblage. Thus, a plant species would have to evolve resistance traits for diverse species of herbivores. In our study, an *Inga* species interacted with an average of 12 different species of lepidopteran herbivores from several distinct families. In addition, our results show that different families of herbivores select hosts based on different defensive traits (Tables 4.3 & 4.4), suggesting that each clade of herbivore might select for the evolution of distinct defensive traits. This scenario is consistent with a model in which plant traits that defend against one herbivore do not correlate with resistance to another (Koskella *et al.* 2012). This appears to happen with *Inga,* where different clades of herbivores respond to distinct plant defenses and where plant defensive traits can diverge independently of each other (e.g., are orthogonal, Table 4.2). This framework suggests that, despite a much shorter generation time for herbivores, plant traits evolve rapidly and that herbivore traits involved in host choice evolve more slowly and depend more on existing host-choice traits (or preadaptations).

This scenario seems consistent with diffuse evolution, a concept that takes into account the fact that interacting species are not in an ecological vacuum, but are connected to other species in the community (Janzen 1980). With respect to plant-insect interactions, this concept specifically predicts that multiple herbivores, are, directly or indirectly, exerting selective pressure on the expression of defensive traits in their hosts (Strauss *et al.* 2005). This kind of diffuse interaction should show a strong geographic

mosaic (Thompson 2005), with species involved in this "multispecies interactions" likely to change from time to time within the same community and from one region to another.

Conclusions

Our results agree with the hypothesis that the interaction between *Inga* and its herbivores might be driving trait evolution, as posited by Ehrlich & Raven (1964). However, our data also suggest that plant defenses might be evolving faster than expected. The fact that closely related *Inga* species present substantial variation in defenses (Kursar *et al.* 2009) in combination with large shifts in defenses between sister *Inga* species (Endara *et al.* 2015), suggests that herbivores may have favored a rapid and divergent evolution of anti-herbivore traits. This view is in strong contrast with the expectations from the coevolutionary theory, which predicts that after the evolution of a key innovation or "a new defense" in response to herbivore pressure, a plant species would be able to radiate into a clade of chemically similar plants. This "key innovation" is assumed to be accomplished through the gain of new genes or new biosynthetic pathways (Berenbaum & Feeny 1981; Berenbaum & Zangerl 1998; Berenbaum & Schuler 2010; Ober 2010). We argue that different mechanisms might be operating. In *Inga*, we have found that the common pattern of chemical novelty is through mix and match of basic building blocks (Kursar *et al.* 2009). Thus, the strategy in *Inga* seems to be a continuous diversification of defense strategies rather than the evolution of a key innovation. Such a pattern is consistent with evolution by changes in gene expression (Wink 2003; Agrawal 2007), a faster and simpler mechanism than would allow for rapid

defense evolution and outpace the relatively short generations times of herbivorous insects.

With respect to herbivore associations, our study suggests that host use in *Inga* is more compatible with a process of ecological tracking rather than with the arms race model of coevolution and cocladogenesis. This process suggests that herbivores associate with novel hosts that express a resource similar to the ancestral host. In other words, herbivores interact with hosts for which their behaviors, morphologies and physiologies are pre-adapted (Janzen 1985; Agosta & Klemens 2008). In this view, coevolutionary history between the herbivore and the host would not be a prerequisite for a successful association (Agosta 2006; Agosta & Klemens 2008; Harvey *et al*. 2012). This interpretation also is consistent with the hypothesis that, despite the short generation time of invertebrate herbivores, plant defenses may evolve more rapidly than the herbivore traits that determine host choice.

Many studies on host-range for insect herbivores at ecological and evolutionary levels are based upon the role of host phylogeny. However, our findings suggest that the associations between plants and insects are more labile than expected under a model of tight evolutionary processes. We argue that for host selection by herbivores, host defenses, or "host resources" (Brooks & McLennan 2002), including food availability, are more important than host phylogeny. Hence, in order to test hypotheses about the role of host range in herbivore ecology and evolution, characterizing host traits is essential.

Growing evidence supports the hypothesis that plant interactions with natural enemies could be a principal mechanism structuring community assembly. Specifically, this theory suggests that divergence in defenses in response to herbivore pressure may

provide a high-dimensionality niche space within which a very large number of co-occurring species might sort in ecological time (Becerra 2007; Kursar *et al.* 2009; Sedio 2013). Kursar *et al.* (2009) reported that co-occurring species of *Inga* in the Peruvian Amazon were more closely related yet differed more in their defenses than expected by chance. Here, we found that lepidopteran herbivores of *Inga* are constrained by differences in defensive traits and thus preferentially forage on subsets of species with similar defensive profiles. Taken together, these results are consistent with density-dependent effects of herbivores on *Inga*. Not sharing herbivores with neighbors gives a species the advantage of reduced damage or "enemy release" (Yguel *et al.* 2011). This in turn may promote the coexistence of species that are closely related but defensively divergent. Studies of other genera in the tropics, *Bursera* and *Psychotria*, reveal the same patterns (Becerra 2007; Sedio 2013). Thus, plant-herbivore interactions may be key to understanding the immense diversity in tropical forest communities.

Table 4.1. Pairwise correlations between defense traits among *Inga* species. Correlation coefficients with † are phylogenetic independent contrasts, the rest are partial mantel *r*. Significant values ($P<0.05$) are in bold

	Trichome length (mm)	Leaf expansion rate (% per day^{-1})	Leaf chlorophyll content (mg per m^{-2})	Mean number of ants per nectary	Ant visitor community to extra-floral nectaries	Chemistry (presence/absence of secondary compounds)	Timing of leaf production (mean angle)	Synchrony in leaf production (cv)
Trichome density (number of hairs per 2 cm^{-2})	**0.79**†	0.1†	-0.07†	-0.08†	0.08	0.01	0.001	0.31†
Trichome length (mm)		-0.06†	-0.15†	-0.05†	0.22	0.07	0.05	-0.2†
Leaf expansion rate (% per day^{-1})			**-0.55**†	0.09†	0.08	0.20	0.05	0.15†
Leaf chlorophyll content (mg per m^{-2})				-0.24†	0.04	0.07	0.002	-0.22†
Mean number of ants per nectary					**0.28**	0.08	0.01	0.14†
Ant visitor community to extra-floral nectaries						0.02	0.03	0.002
Chemistry (presence/absence of secondary compounds)							0.06	0.03
Timing of leaf production (mean angle)								0.04

Table 4.2. PCA loadings. Correlations between components and continuous defense traits.

	Trichome density	Trichome lenght	Leaf expansion rate	Leaf chlorophyll content	Mean number of ants per nectary	Synchrony in leaf production (CV)
Component 1	0.58	0.60	-0.33	0.24	0.09	-0.38
Component 2	0.25	0.18	0.60	-0.69	0.09	-0.20
Component 3	0.04	0.07	-0.20	-0.17	0.85	0.40
Component 4	-0.20	-0.26	0.29	0.36	0.50	-0.70

Table 4.3. Summary statistics for the relationship between herbivore communities and host plant traits. *r* represents the Mantel correlation between the dissimilarity in host plant traits and their herbivore communities measured by the Bray-Curtis index. Significant values ($P<0.05$) are in bold

Host plant traits	All herbivores	Elachistidae	Riodinidae	Erebidae
	r	*r*	*r*	*r*
Phylogeny	**0.25**	**0.24**	0.2	0.04
All defenses	**0.5**	**0.42**	**0.33**	0.16

Table 4.4. Results of best-fit distance-based redundancy analyses (db-RDA) model for the three most abundant lepidopteran families

Parameter	R^2_{adj}	P
Elachistidae		
Full model	0.42	0.012
Chemistry	0.23	0.015
Developmental	0.06	0.012
Phylogeny	0.14	0.011
Erebidae		
Full model	0.25	0.023
Chemistry	0.14	0.019
Synchrony in leaf production (CV)	0.04	0.2
Timing of leaf production (mean angle)	0.13	0.04
Riodinidae		
Full model	0.40	0.002
Biotic	0.29	0.014
Chemistry	0.27	0.04
Physical	0.12	0.06

Table 4.5. Results of maximum likelihood analyses for the three most abundant lepidopteran families against host plant traits. AICc: Akaike Information Criteria corrected for small sample sizes. ΔAICc: difference in AIC scores between the best model (listed first) and each competing model. *wi*: AIC weight (level of support for a model, max. weight=1). Intercept: null model. Coefficient estimates: raw estimates that indicate a positive or negative effect of each predictor variable on the response variable. Values of coefficients whose 95% credible interval (95% CI) does not include zero are in bold.

Herbivore	Predictor Variable	*Df*	ΔAICc	w_i	Coefficient estimate (95% CI)
Elachistidae	Phylogeny+ phenolics+saponins+phenolics and saponins+amines and phenolics+trichome length+trichome number+leaf expansion rate	9	0	0.67	**0.1 (0.2 to 1.0)** + 0.01 (0.7 to -0.7) + 1.02 (2.1 to -0.02) + **1.6 (2.5 to 0.7)** + **-0.6 (-0.02 to -1.33)** + -0.36 (0.04 to 0.8) + 0.2 (0.36 to 0.04) + **0.9(1.4 to 0.3)**
	Intercept	1	60.6	<0.001	
Riodinidae	Mean number of ants + leaf expansion rate	3	0	0.35	**3.09 (5.4 to 1)** + 0.66 (1.6 to -0.22)
	Intercept	1	6.9	0.01	
Erebidae	Timing of leaf production +Synchrony in leaf production+ amines and phenolics	4	0	0.4	**-0.22 (-0.01 to -0.4)** + 0.85(1.72 to -0.020) + **0.86 (1.6 to 0.1)**
	Intercept	1	6.5	0.01	

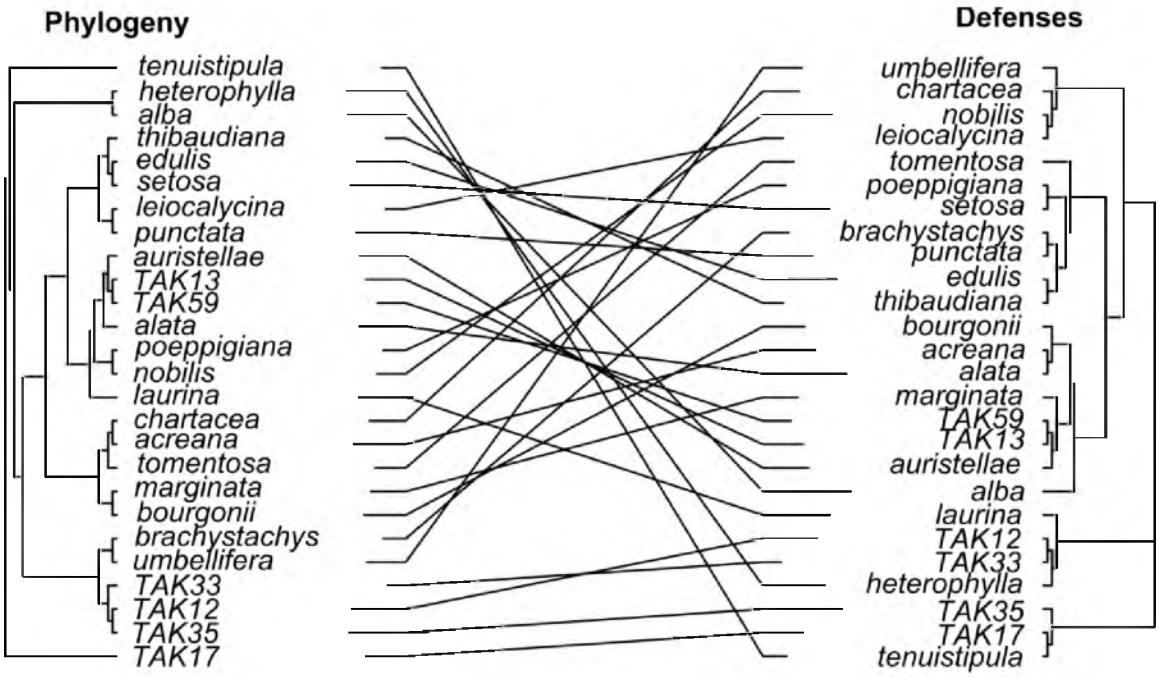

Figure 4.1. Comparison between the phylogenetic tree (left) and the defensogram (defense dendrogram, right) for *Inga* species.

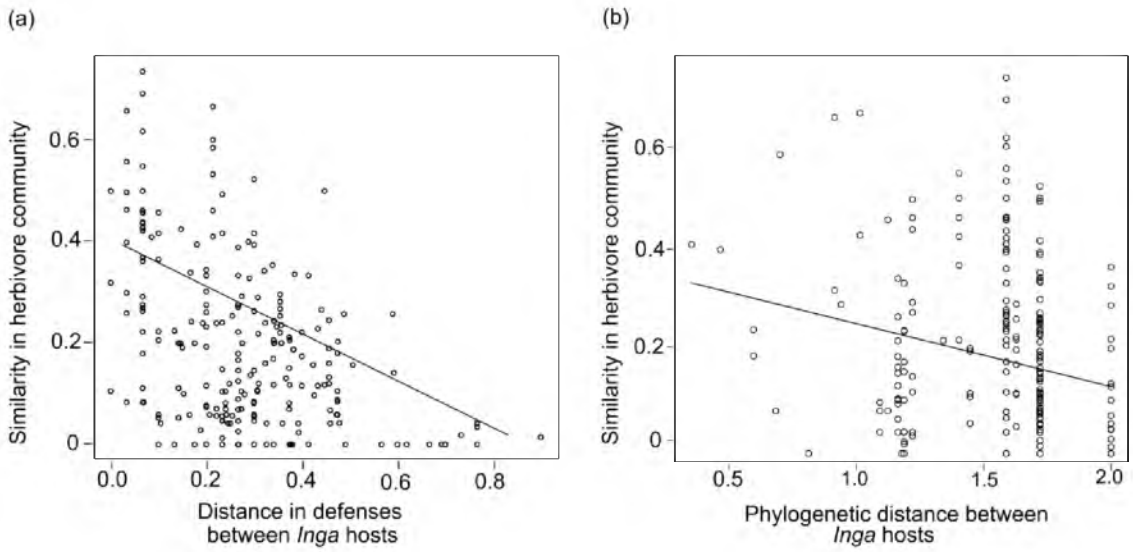

Figure 4.2. Relationship between the similarity of lepidopteran communities (based on Bray-Curtis Index) on host plants vs. (a) distance in defenses between *Inga* hosts and (Partial Mantel r= 0.5, p=0.001) (b) phylogenetic distance between *Inga* hosts for all pairwise combinations of plants (Mantel r= 0.25, p=0.02).

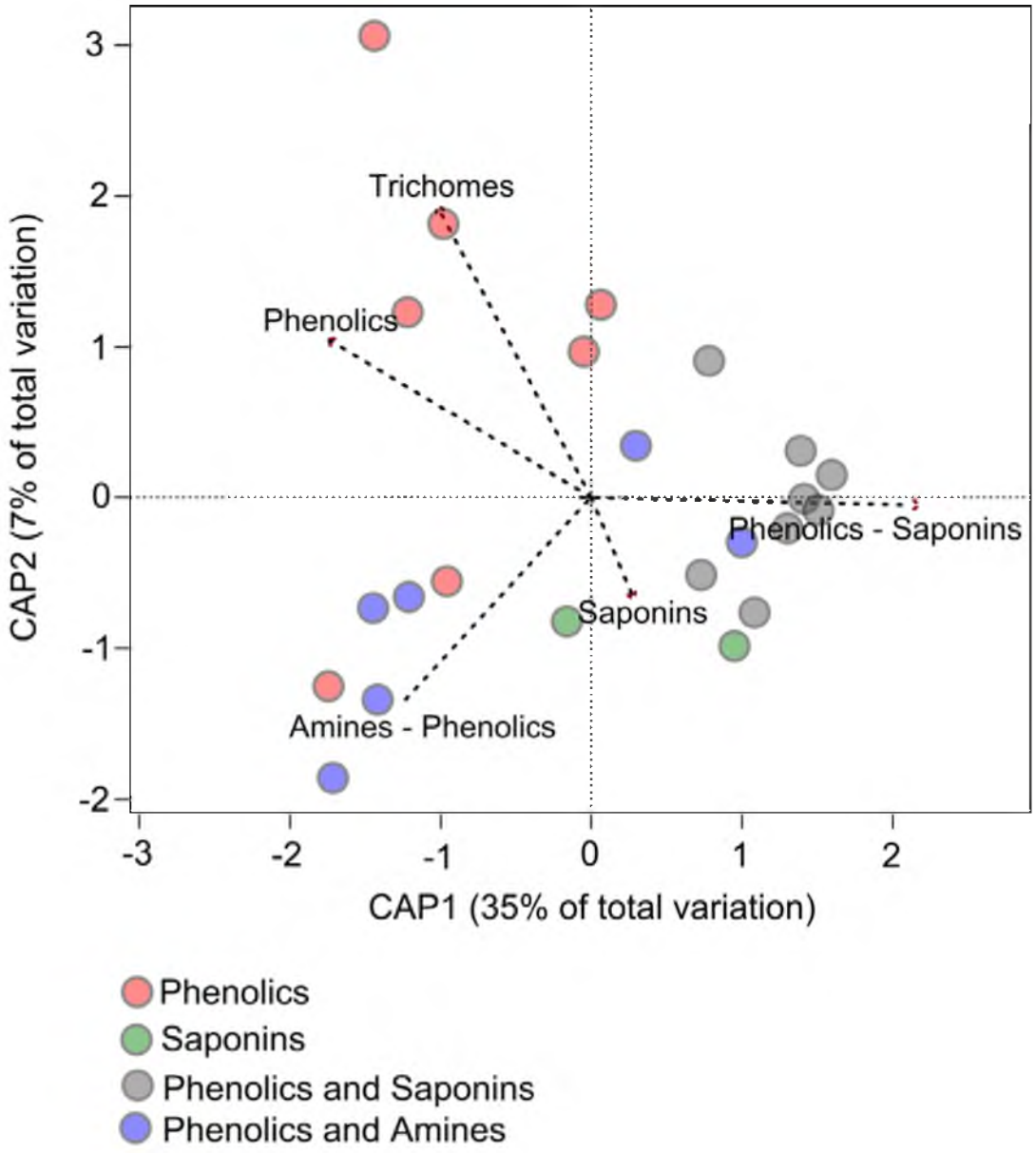

Figure 4.3. Constrained Analysis of Principal Coordinates of the most parsimonious model for the lepidoptera community similarity measured by the Bray-Curtis index (R^2_{adj}= 0.4, p=0.001). Each dot represents an *Inga* species host color-coded by defense chemistry.

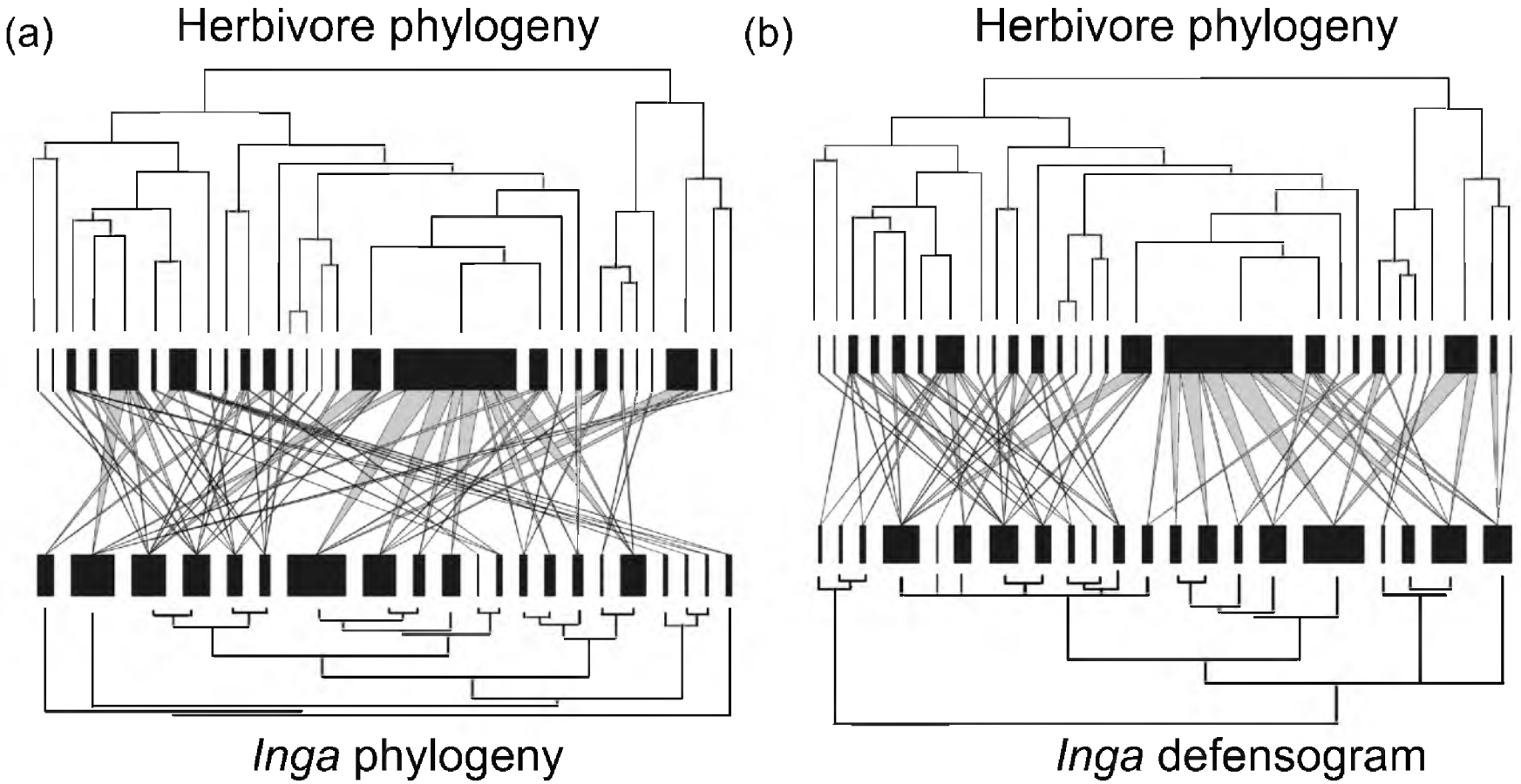

Figure 4.4. Bipartite trophic network of *Inga* hosts and herbivores. (a) Phylogenies of *Inga* and Elachistidae plotted in the margins (Parafit test: p=0.52). (b) Phylogeny of Elachistidae and *Inga* defensogram plotted in the margins (Parafit test: p=0.05). For each network lower bars represent host abundance and upper bars represent herbivores abundance. Linkage width represents frequency of the interaction.

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APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 3

Appendix A.1. Photographs of the *Inga* species complexes. (a) *Inga capitata* ESU cap1, (b) *Inga capitata* ESU cap2, (c) *Inga capitata* ESU cap3, (d) *Inga heterophylla* ESU het1 and (e) *Inga heterophylla* ESU het2.

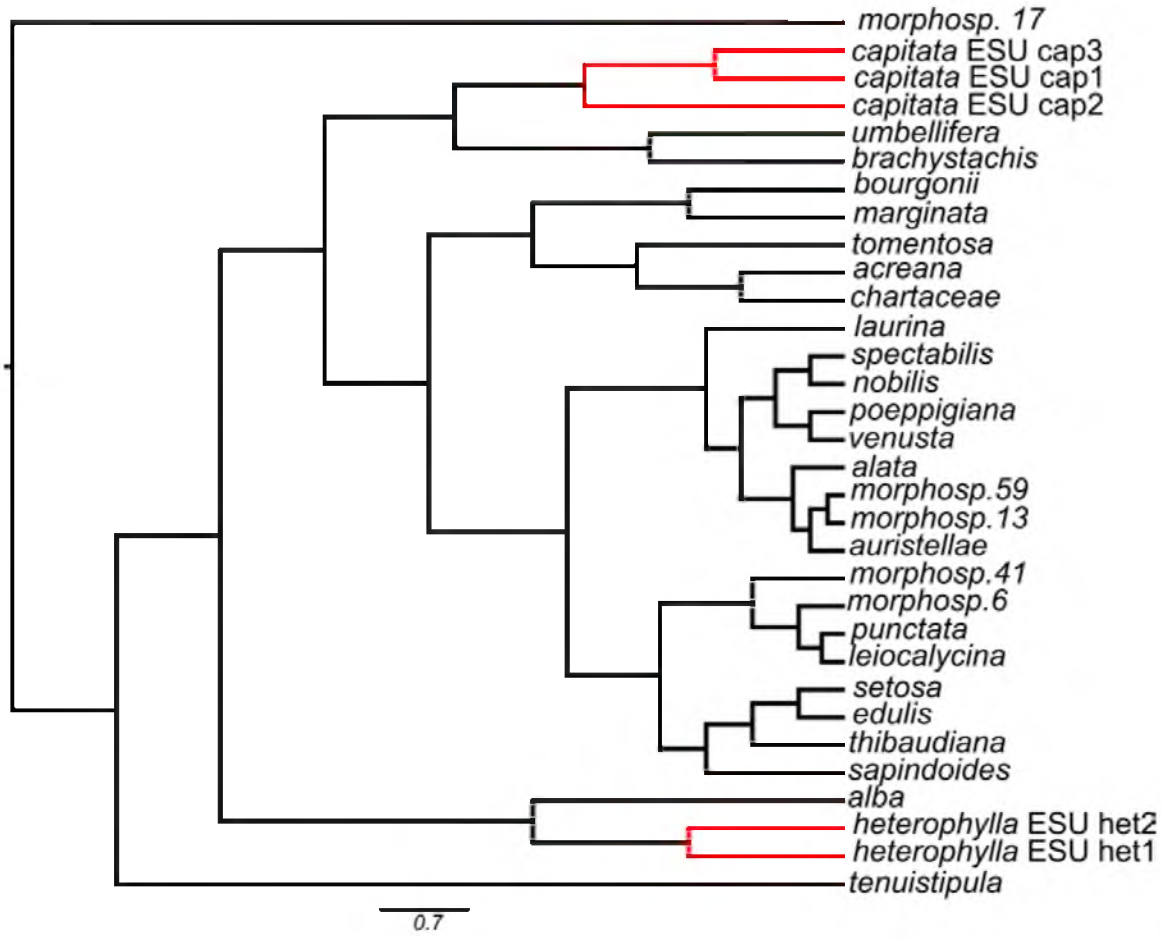
(a)

(b)

(c)

(d)

(e)



Appendix A.2. Maximum clade credibility tree of the species of the genus *Inga* in Los Amigos, Peru, from a Bayesian analysis (in BEAST, Drummond & Raumbaut. 2007) of 6000 bp of plastid DNA and the ITS nuclear marker. Branch lengths represent time in millions of years. For the root node, a normally distributed prior was used with a mean of six million years based on divergence times across legumes. Clades containing the *I. capitata* species complex (ESUs cap1, cap2 and cap3) and *I. heterophylla* species complex (ESUs het1, het2) are colored in red. This tree shows that the ESUs within each species complex are more closely related to each other than with any other *Inga* species (Modified from Kursar *et al.* 2009 by Dr. Kyle Dexter).

Appendix A.3. Chromatographi gradient used for the LC-MS analyses

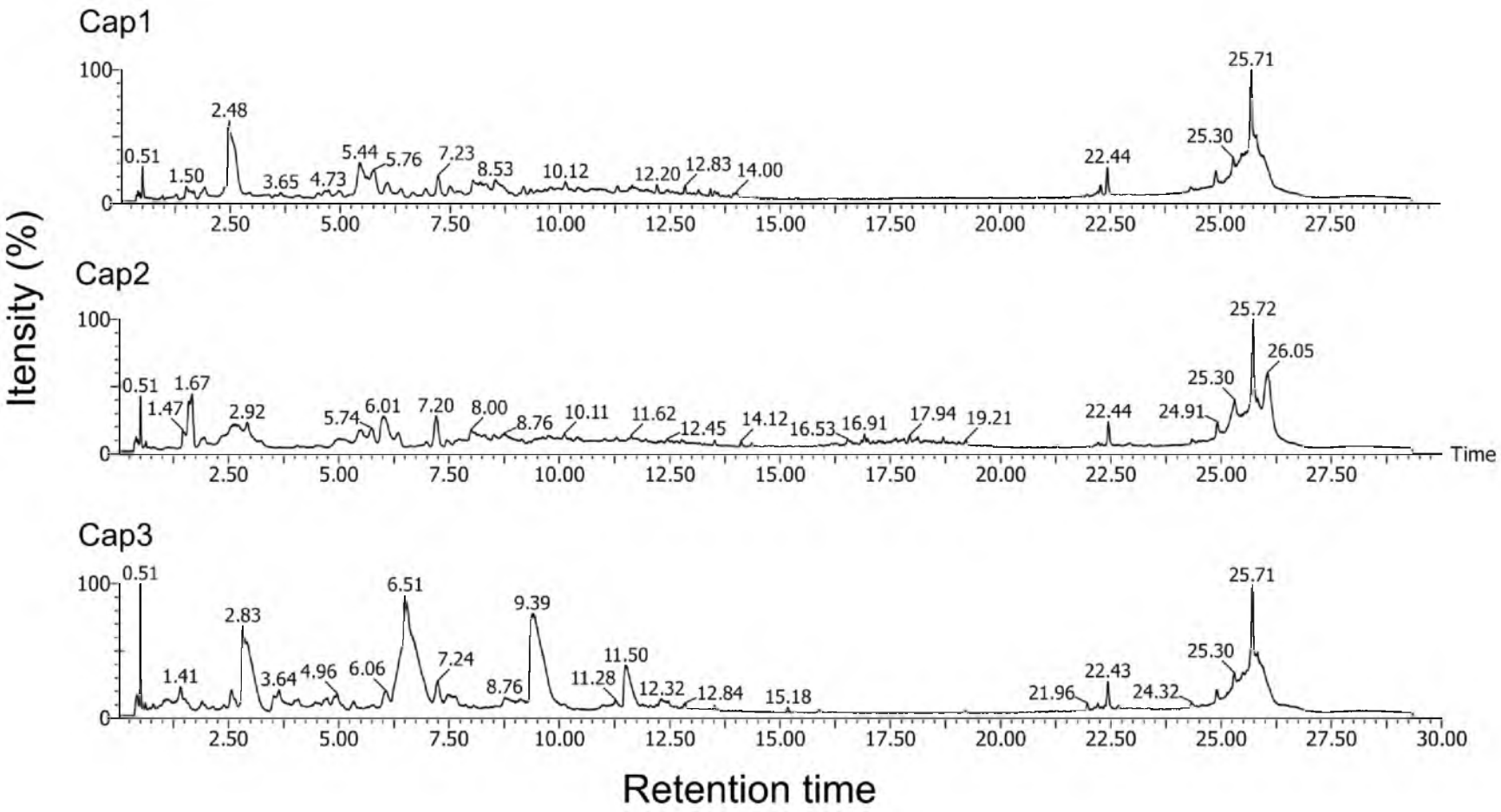
Time (min)	Solvent A (%)	Solvent B (%)
1	95	5
10	85	15
20	50	50
25	5	95
30	95	5
35	95	5

Appendix A.4. Phenolic content for *Inga* species as g phenolics per g DW of leaf (± 1 SD) for 5 replicate extractions. Analyses of Variance did not detect differences in phenolic investment among the ESUs within each species complex.

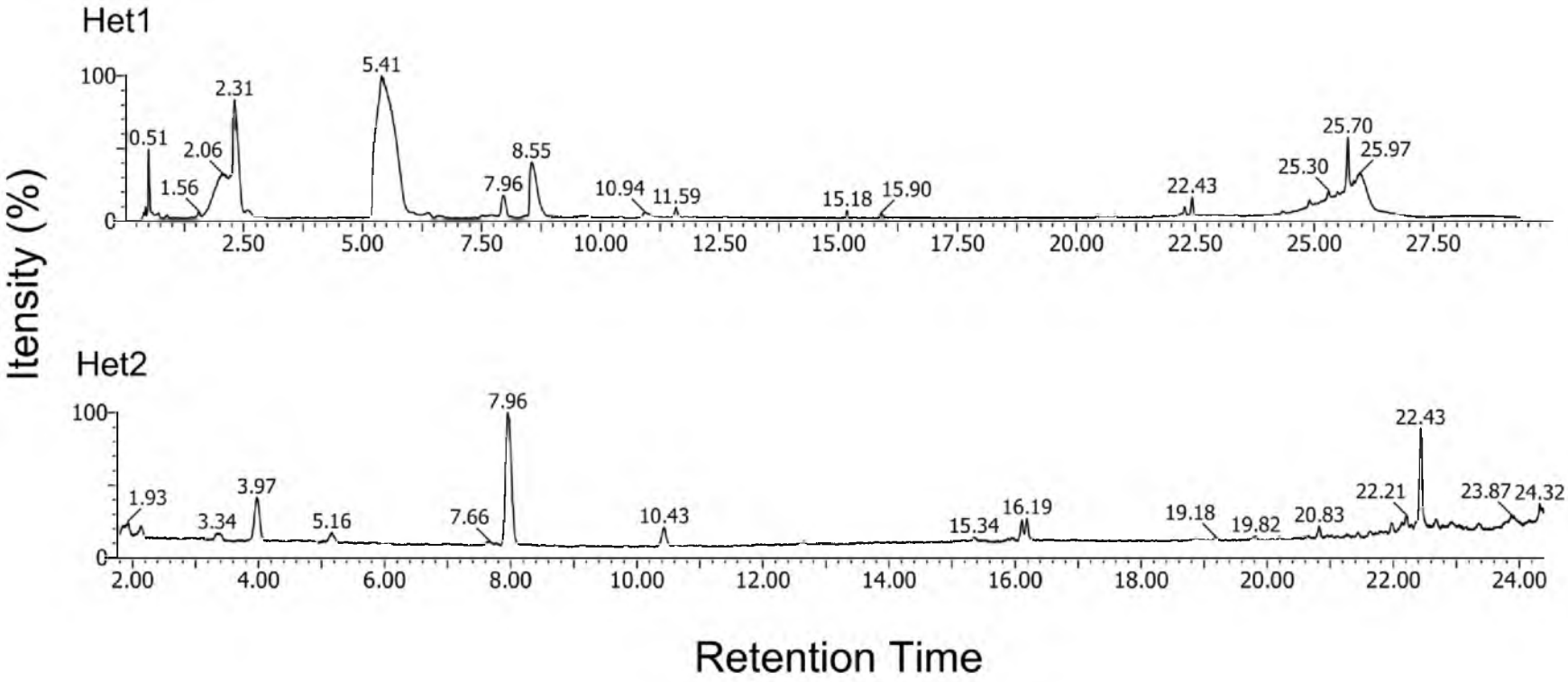
Inga capitata species complex			*Inga heterophylla* species complex	
cap1	cap2	cap3	het1	het2
0.39 ± 0.15	0.36 ± 0.03	0.32 ± 0.06	0.23 ± 0.04	0.17 ± 0.09

Appendix A.5. Total ion chromatograms showing relative intensities of peaks from the LC-QToF-MS for the different ESUs in (a) and (b) positive mode and (c) and (d) negative mode. (a) and (c) *I. capitata* species complex, (b) and (d) *I. heterophylla* species complex.

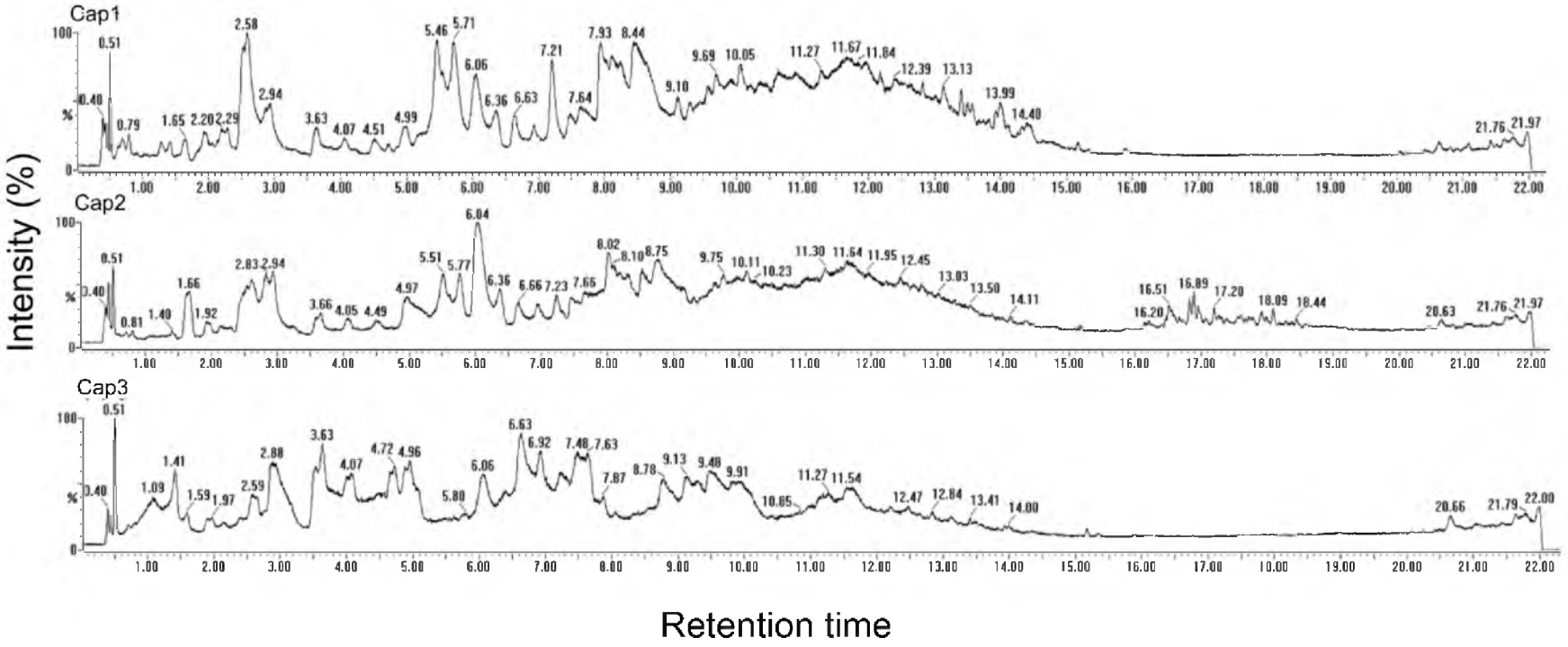
(a)


Cap1
Cap2
Cap3
Itensity (%)
Retention time
Time
100
0
0.51
1.50
2.48
3.65
4.73
5.44
5.76
7.23
8.53
10.12
12.20
12.83
14.00
22.44
25.30
25.71
1.47
1.67
2.92
5.74
6.01
7.20
8.00
8.76
10.11
11.62
12.45
14.12
16.53
16.91
17.94
19.21
24.91
25.72
26.05
1.41
2.83
3.64
4.96
6.06
6.51
7.24
8.76
9.39
11.28
11.50
12.32
12.84
15.18
21.96
22.43
24.32
2.50
5.00
7.50
10.00
12.50
15.00
17.50
20.00
22.50
25.00
27.50
30.00

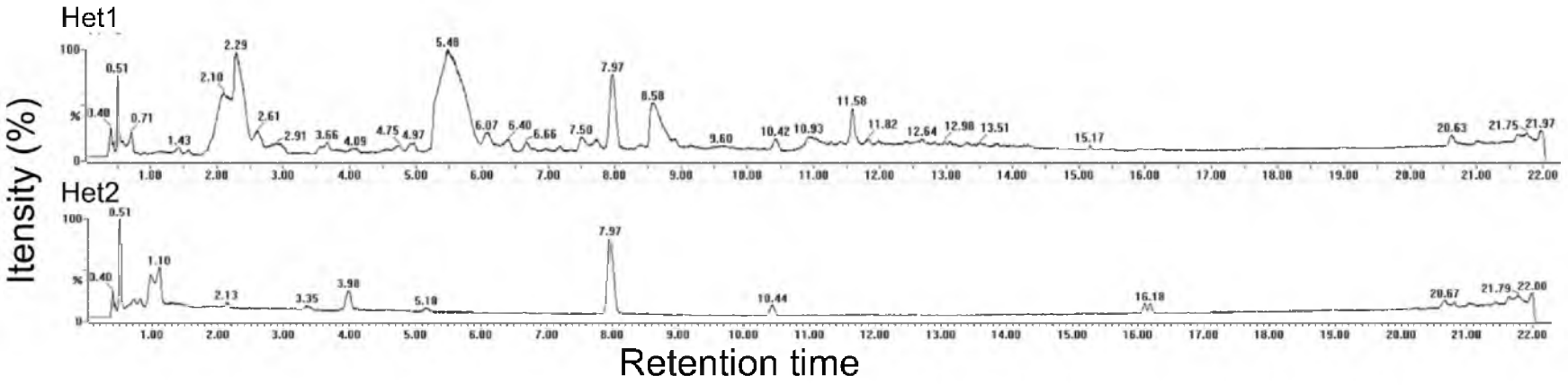
(b)


Het1
100
0
0.51
1.56
2.06
2.31
5.41
7.96
8.55
10.94
11.59
15.18
15.90
22.43
25.30
25.70
25.97
2.50
5.00
7.50
10.00
12.50
15.00
17.50
20.00
22.50
25.00
27.50
Het2
100
0
1.93
3.34
3.97
5.16
7.66
7.96
10.43
15.34
16.19
19.18
19.82
20.83
22.21
22.43
23.87
24.32
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
Itensity (%)
Retention Time

(c)



(d)



Appendix A.6. Metabolites that distinguish the ESUs (biomarkers) were detected by LC-QToF-MS and were identified by PCA or by inspection. The mass to charge ratio of the ion is indicated as "*m/z*". "Elemental composition" was obtained from MassLynx (Elemental Composition v 4.0©, Waters Corporation, 2000, Milford, MA). The "expected *m/z*" equals the monoisotopic mass calculated from "elemental composition" by MassLynx or from isotopic masses in the National Institute of Standards and Technology database (Coursey et al. 2010). The error in the observed *m/z* is in parts per million (ppm). "GC/EG" is gallocatechin/epigallocatechin. "DOPA" is 3,4-dihydroxyphenylalanine. The columns for cap1, cap2, cap3, het1, and het2 indicate the approximate abundance of each *m/z* in exponential notation or the approximate abundance as follows: moderately abundant (>10^5 ion counts), abundant (10^4 to 10^5 ion counts), and present (10^3 to 10^4 ion counts) indicated as "xx", "x", and "p," respectively. A question mark indicates detected in 1 to 3 samples and missing in 2 to 4 samples.

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion	Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
GC/EG	1.00 to 1.10	307.0795, POS	$[C_{15}H_{14}O_7+H]^+$	307.0812	5.6	nd	nd	nd	nd	2e4
monomer	0.99 to 1.10	305.0661, NEG	$[C_{15}H_{14}O_7-H]^-$	305.0667	1.9	nd	nd	nd	4e3	1e5
Unknown	1.21	136.0744, POS	$[C_8H_9NO+H]^+$	136.0757	9.5	3e4	2e3	p	nd	nd
		NEG		134.0611		nd	nd	nd	nd	nd
Unknown	1.27	160.0745, POS	$[C_{10}H_9NO+H]^+$	160.0757	7.4	7e4	9e3		nd	nd
		NEG		158.0611		nd	nd	nd	nd	nd
Di-O galloyl-quinic acid	1.41	495.0769, NEG	$[C_{21}H_{20}O_{14}-H]^-$	495.0780	2.3	5e4	nd	2e5	1e4	nd

Appendix A.6. (continued)

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion
Mono-O-	1.56	350.0857, POS	$[C_{16}H_{15}NO_8+H]^+$
galloylDOPA		348.0712, NEG	$[C_{16}H_{15}NO_8 - H]^-$
Unknown	1.66	206.0799, POS	$[C_{11}H_{11}NO_3+H]^+$
		NEG	
Unknown	1.97	236.1267, POS	$[C_{13}H_{17}NO_3+ H]^+$
		NEG	
GC/EG dimer	2.15	611.1389, POS	$[C_{30}H_{26}O_{14}+ H]^+$
		609.1234, NEG	$[C_{30}H_{26}O_{14} - H]^-$
Mono-O-	2.28	334.0910	$[C_{16}H_{15}NO_7+ H]^+$
galloyltyrosine	2.27	332.0768	$[C_{16}H_{15}NO_7 - H]^-$
Unknown	2.38	144.0802, POS	$[C_{10}H_9N + H]^+$
		NEG	
Mono-O-galloyltyrosine	2.35 to 2.45	332.0775, NEG	$[C_{16}H_{15}NO_7 - H]^-$

Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
350.0870	3.8	nd	nd		4e5	nd
348.0725	3.7	nd	nd	nd	2e3	nd
206.0812	6.2	3e4	5e5		nd	nd
		nd	nd	nd	nd	nd
236.1281	5.9	2e4	3e4		nd	nd
		nd	nd	nd	nd	nd
611.1395	1.0	nd	nd		nd	9e3
609.1250	2.6	nd	nd	nd	nd	9e3
334.0921	3.3	5e3	nd	x	1e6	nd
332.0776	2.3	2e3	2e3	4e3	3e5	nd
144.0808	4.0	2e6		x	nd	nd
		nd	nd	nd	nd	nd
332.0776	0.0	nd	5e3	4e3	nd	nd

Appendix A.6. (continued)

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion
Di-*O*-galloylquinic acid	2.54	495.0784, NEG	$[C_{21}H_{20}O_{14} - H]^-$
Di-*O*-galloyl-quinic acid	2.60	497.0912, POS	$[C_{21}H_{20}O_{14}+H]^+$
	2.55	495.0779, NEG	$[C_{21}H_{20}O_{14} - H]^-$
Digallate	2.61	321.0243, NEG	$[C_{14}H_{10}O_9 - H]^-$
Unknown	2.66	144.0797, POS	$[C_{10}H_9N + H]^+$
		NEG	
Di-*O*-galloylquinic acid	2.77	495.0782, NEG	$[C_{21}H_{20}O_{14} - H]^-$
Mono-O-galloyltyramine	2.73	290.1014, POS	$[C_{15}H_{15}NO_5 + H]^+$
	2.73	288.0882, NEG	$[C_{15}H_{15}NO_5 - H^+]^-$

Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
495.0780	0.1	1e5	1e5	5e4	9e3	nd
497.0926	2.8	3e4	4e4	xx	nd	nd
495.0780	0.2	2e5	1e5	5e4	x	nd
321.0252	2.8	9e4	9e4	1e5	5e4	nd
144.0808	7.6	xx	2e5			
		nd	nd	nd	nd	nd
495.0780	0.4	nd	1e5	1e5	1e4	nd
290.1023	3.1	p	x	5e5	nd	nd
288.0877	1.6	nd	nd	xx	nd	nd

Appendix A.6. (continued)

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion
Di-*O*-galloylquinic acid	2.86	495.0787, NEG	$[C_{21}H_{20}O_{14} - H]^-$
Di-*O*-galloylquinic acid	2.93	497.0931, POS	$[C_{21}H_{20}O_{14}+H]^+$
		495.0772, NEG	$[C_{21}H_{20}O_{14} - H]^-$
Unknown	3.39	217.1320, POS	
		NEG	
Tri-*O*-galloylquinic acid	3.63	649.1033, POS	$[C_{28}H_{24}O_{18} + H]^+$
		647.0872, NEG	$[C_{28}H_{24}O_{18} - H]^-$
Unknown	4.23	307.0661, POS	
		465.1022, NEG	$[C_{21}H_{22}O_{12} - H]^-$

Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
495.0780	1.4				1e4	nd
497.0926	1.0		6e4			
495.0780	1.7	nd	1e5	6e4	1e4	nd
		6e4	5e3		nd	nd
		nd	nd	nd	nd	nd
649.1035	0.3	4e3	?	xx	2e3	nd
647.0872	2.8	8e4	nd	3e5	2e4	nd
		1e4	?			nd
465.1039	3.5	2e4	nd	7e3	nd	nd

Appendix A.6. (continued)

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion
Tri-*O*-galloylquinic acid	5.02 4.95	POS 647.0878, NEG	$[C_{28}H_{24}O_{18} - H]^-$
Unknown	5.42	215.1158, POS NEG	
Di_*O*-gallolyltyrosine	5.43	484.0882, NEG	$[C_{23}H_{19}NO_{11} - H]^-$
Unknown	5.45	969.1838, NEG	$[C_{46}H_{38}N_2O_{22}-H]^-$
Tri-*O*-galloylquinic acid	5.51	647.0880, NEG	$[C_{28}H_{24}O_{18} - H]^-$
Tri-*O*-galloyl-quinic acid	5.76	647.0892, NEG	$[C_{28}H_{24}O_{18} - H]^-$

Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
647.0890	1.8	7e4	6e4	2e5	1e4	nd
		1e4	x		nd	nd
		nd	nd	nd	nd	nd
484.0885	0.7	nd	nd	nd	xx	nd
969.1838	0.6	nd	nd	nd	x	nd
647.0890	1.5	3e5	2e5	2e5	nd	nd
647.0890	0.3	3e5	2e5	3e4	2e3	nd

Appendix A.6. (continued)

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion
Tri-*O*-galloylquinic acid	6.08	647.0895, NEG	$[C_{28}H_{24}O_{18} - H]^-$
Di-O-galloyltyramine	6.52	442.1124, POS	$[C_{22}H_{19}NO_9 + H]^+$
	6.28 to 6.49	440.0988, NEG	$[C_{22}H_{19}NO_9 - H]^-$
Tetra-O-galloylquinic acid	6.63	799.1019, NEG	$[C_{35}H_{28}O_{22} - H]^-$
Unknown	6.68	601.1161, POS NEG	$[C_{28}H_{24}O_{15} + H]^+$
Tetra-O-galloylquinic acid	6.92	799.1017, NEG	$[C_{35}H_{28}O_{22} - H]^-$

Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
647.0890	0.8	1e5	2e5	9e4	2e4	
442.1133	1.9	nd	5e4	xx	nd	nd
440.0987	0.2	nd	nd	2e5	nd	nd
799.0999	2.5	5e4	nd	1e5	3e3	nd
601.1188	4.5	2e4	x		nd	nd
		nd	nd	Nd	nd	nd
799.0999	2.3	4e4	nd	2e5	1e3	nd

Appendix A.6. (continued_

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion
Di-O-galloyltyramine	7.01	440.0999, NEG	$[C_{22}H_{19}NO_9 - H]^-$
Unknown	7.22	291.0954, POS	
		NEG	
Tetra-O-galloylquinic acid	7.9 to 8.1	801.1141, POS	
		799.1014, NEG	$[C_{35}H_{28}O_{22} - H]^-$
Unknown	7.97	487.0840, POS	$[C_{21}H_{20}O_{12}+Na]^+$
		463.0875, NEG	$[C_{21}H_{20}O_{12} - H]^-$
Tetra-O-galloylquinic acid	8.31	799.1004, NEG	$[C_{35}H_{28}O_{22} - H]^-$
Tetra-O-galloyl-quinic acid	8.52	799.1013, NEG	$[C_{35}H_{28}O_{22} - H]^-$

Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
440.0987	2.7	2e3	3e4	3e4	nd	nd
		1e4	xx	xx	nd	nd
		nd	nd	nd	nd	nd
		5e3	x	xx	nd	nd
799.0999	1.9	2e5	2e5	2e4	nd	nd
487.0847	1.4	3e3			5e4	3e4
463.0882	1.5	nd	nd	nd	2e5	2e5
799.0999	0.6	2e5	1e5	nd	nd	nd
799.0999	1.8	2e5	1e5	nd	nd	nd

Appendix A.6. (continued)

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion
Tri-*O*-gallolyltyrosine	8.57	636.0993, NEG	$[C_{30}H_{23}NO_{15} - H]^-$
Tetra-O-galloylquinic acid	8.73	799.1001, NEG	$[C_{35}H_{28}O_{22} - H]^-$
Tri-O-galloyltyramine	9.43	594.1242, POS	$[C_{29}H_{23}NO_{13} + H]^+$
	9.40	592.1101, NEG	$[C_{29}H_{23}NO_{13} - H]^-$
Tetragallate	9.55	625.0453, NEG	$[C_{28}H_{18}O_{17} - H]^-$
Unknown	10.11	657.1091, NEG	$[C_{30}H_{26}O_{17} - H]^-$
Quercetin-de-oxyhexose	10.43	471.0866	$[C_{21}H_{20}O_{11} + Na]^+$
	10.43	447.0923	$[C_{21}H_{20}O_{11}\text{-}H^+]^-$
Tetra-*O*-galloyltyrosine	10.99	788.1097, NEG	$[C_{37}H_{27}NO_{19}\text{-}H]^-$

Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
636.0995	0.3	nd	nd	nd	2e5	nd
799.0999	0.3	1e5	1e5	4e4	2e3	nd
594.1242	0.0	p	x	xx	nd	nd
592.1097	0.7	nd	5e3	xx	nd	nd
625.0471	2.9	x	8e4	x	2e4	nd
657.1097	0.9	xx	7e4	P	nd	nd
471.0898	6.8	3e3	p	p	6e3	?e3
447.0933	2.2	7e3	6e4	3e4	x	x
788.1105	1.0	nd	nd	nd	x	nd

Appendix A.6. (continued)

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion
Hexa-O-galloylquinic acid	11.46	1105.1378, POS	$[C_{49}H_{36}O_{30}+H]^+$
		1103.1211, NEG	$[C_{49}H_{36}O_{30}-H]^-$
Pentagallate	11.62	777.0576, NEG	$[C_{35}H_{22}O_{21}-H]^-$
Hexa-O-galloylquinic acid	11.89	1103.1218, NEG	$[C_{49}H_{36}O_{30}-H]^-$
Unknown	11.99	745.1042, NEG	$[C_{36}H_{26}O_{18}-H]^-$
Hexagallate	12.17	929.0689, NEG	$[C_{42}H_{26}O_{25}-H]^-$
Unknown	12.42	745.1063, NEG	$[C_{36}H_{26}O_{18}-H]^-$
Hexagallate	12.47	929.0685, NEG	$[C_{42}H_{26}O_{25}-H]^-$
Unknown	12.82	745.1048, NEG	$[C_{36}H_{26}O_{18}-H]^-$
Unknown	12.93	745.1038, NEG	$[C_{36}H_{26}O_{18}-H]^-$

Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
1105.1364	1.3	2e4	1e4	x	nd	nd
1103.1219	0.7	5e4	3e4	1e4	nd	nd
777.0581	0.6	4e4	5e4	x		
1103.1219	0.1	5e4	3e4	3e3	nd	nd
745.1046	0.6	1e4	nd	1e4	nd	nd
929.0690	0.2	6e3	3e4	4e4	nd	nd
745.1046	2.2	4e4	nd	4e4	nd	nd
929.0690	0.5	2e4	2e4	7e3	nd	nd
745.1046	0.2	1e5	6e3	1e5	nd	nd
745.1046	1.1	3e4	nd	3e4	nd	nd

Appendix A.6. (continued)

Putative IDs	Retention time (min)	Observed *m/z*	Elemental composition of observed ion	Expected *m/z*	Error (ppm)	cap1	cap2	cap3	het1	het2
Unknown	13.03	745.1035, NEG	$[C_{36}H_{26}O_{18}\text{-}H]^-$	745.1046	1.5	4e4	3e3	5e4	nd	nd
Unknown	13.13	745.1039 NEG	$[C_{36}H_{26}O_{18}\text{-}H]^-$	745.1046	1.0	1e5	2e3	9e4	nd	nd
Unknown	13.41	745.1035, NEG	$[C_{36}H_{26}O_{18}\text{-}H]^-$	745.1046	1.5	8e4	4e3	8e4	nd	nd
$(GC/EG)_2$-	14.16	915.1699, POS	$[C_{44}H_{34}O_{22}+H]^+$	915.1614	9.2	nd			1e3	nd
digallate		NEG		913.1469		nd	nd	nd	nd	nd
Unknown	21.27	258.2784, POS	$[C_{16}H_{35}NO+ H]^+$	258.2791	2.9	4e4	x	x	5e3	nd
		NEG				nd	nd	nd	nd	nd

Appendix A.7. Insect herbivore species found on each *I. capitata* ESU. Total number of insects observed by ESU are indicated. For the analysis of herbivore association, singletons were not included.

Order	Family	Subfamily	Genus species	cap1	cap2	cap3
Hymenoptera	Argidae		MOTU0175	10	77	48
Lepidoptera	Erebidae	Erebinae	*Coenipeta* MOTU0108	2	2	8
Coleoptera	Chrysomelidae	Eumolpinae	*Eumolpinae* sp.1	8	1	0
Lepidoptera	Elachistidae		MOTU0019	0	7	0
Lepidoptera	Elachistidae	Stenomatinae	*Antaeotrichia* MOTU0004	2	1	3
Lepidoptera	Elachistidae	Stenomatinae	*Antaeotrichia* MOTU0078	4	0	1
Coleoptera	Curculionidae	Leptopinae	*Leptopinae* sp.1	0	3	0
Lepidoptera	Erebidae	Erebinae	*Letys micerina* MOTU0043	0	1	1
Lepidoptera	Lycaenidae	Techlinae	*Symbiopsis tanais* MOTU0044	2	0	0
Lepidoptera	Elachistidae	Stenomatinae	*Antaeotrichia* MOTU0049	0	2	0

Appendix A.7. (continued)

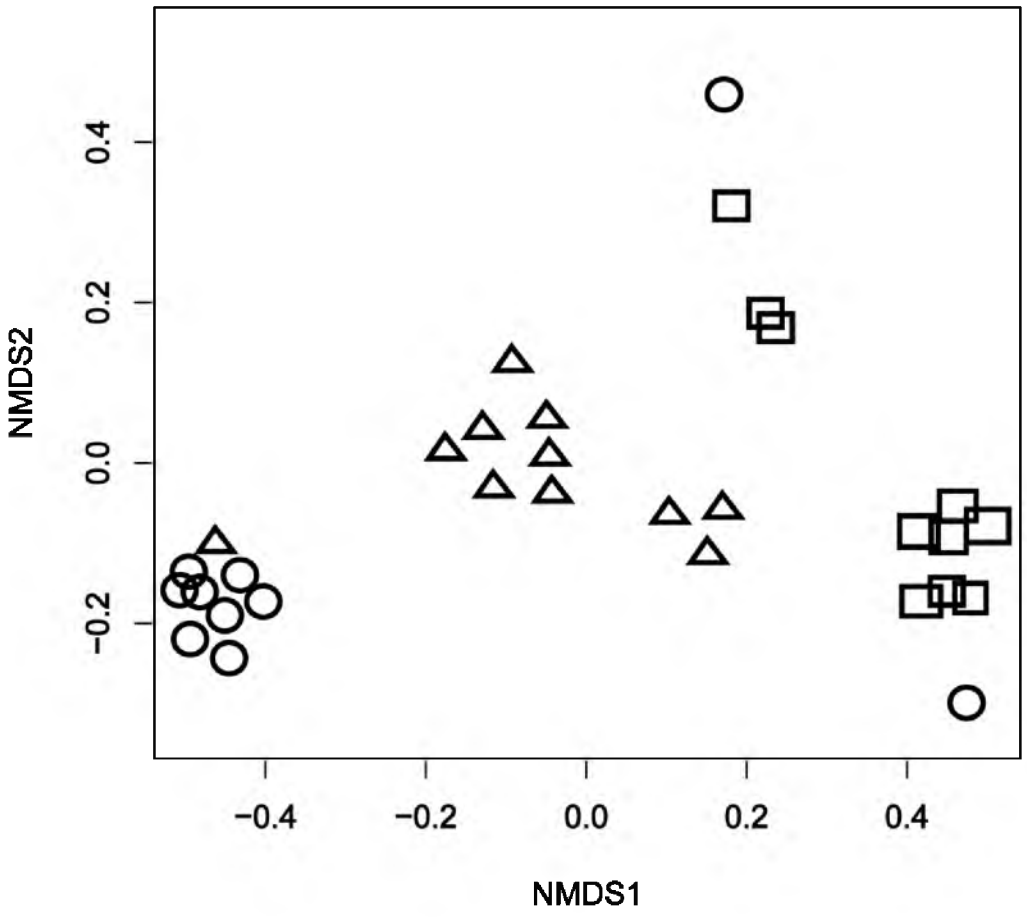
Order	Family	Subfamily	Genus species	cap1	cap2	cap3
Lepidoptera	Elachistidae		MOTU0060	0	0	2
Lepidoptera	Erebidae	Erebinae	*Helia exsicatta* MOTU0065	1	0	1
Lepidoptera	Erebidae	Arctiinae	*Melese* MOTU0021	0	0	1
Lepidoptera	Elachistidae	Stenomatinae	MOTU0024	0	0	1
Lepidoptera	Geometridae	Ennominae	*Paragonia tassima* MOTU0032	1	0	0
Lepidoptera	Crambidae	Spilomelinae	MOTU0033	1	0	0
Lepidoptera	Notodontidae	Hemiceratinae	*Hemiceras nigrescens* MOTU0047	0	0	1
Lepidoptera	Elachistidae		MOTU0050	0	0	1
Lepidoptera	Tortricidae	Tortricinae	*Amorbia productana* MOTU0059	0	1	0
Lepidoptera	Erebidae	Arctiinae	*Talara semiflava* MOTU0069	1	0	0
Lepidoptera	Elachistidae		MOTU0076	0	0	1

Appendix A.7. (continued)

Order	Family	Subfamily	Genus species	cap1	cap2	cap3
Lepidoptera	Geometridae	Ennominae	*Paragonia cruraria* MOTU0094	0	0	1
Lepidoptera	Lycaenidae	Techlinae	*Theritas hemon* MOTU0100	0	1	0
Lepidoptera	Erebidae	Arctiinae	MOTU0101	0	0	1
Lepidoptera	Elachistidae	Stenomatinae	*Antaeotrichia* MOTU0102	0	1	0
Lepidoptera	Erebidae	Arctiinae	*Ormetica rothschildi* MOTU0119	0	1	0
Lepidoptera	Erebidae	Eulepidotinae	*Antiblema* MOTU0128	0	1	0
Lepidoptera	Geometridae	Ennominae	*Iridopsis herse* MOTU0129	0	0	1
Lepidoptera	Geometridae	Ennominae	*Hypometalia* MOTU0137	0	1	0
Lepidoptera	Erebidae	Arctiinae	*Melese castrena* MOTU0140	0	0	1

Appendix A.7. (continued)

Order	Family	Subfamily	Genus species	cap1	cap2	cap3
Lepidoptera	Geometridae	Ennominae	*Paragonia* MOTU0151	0	1	0
Lepidoptera	Geometridae	Geometrinae	*Ishnopteris* MOTU0160	0	1	0
Coleoptera	Curculionidae	Brachyderinae	Brachyderinae sp.1	0	1	0
Coleoptera	Ptilodactylidae		Ptilodactylidae sp.1	0	1	0
Coleoptera	Curculionidae		Curculionidae sp.1	0	0	1



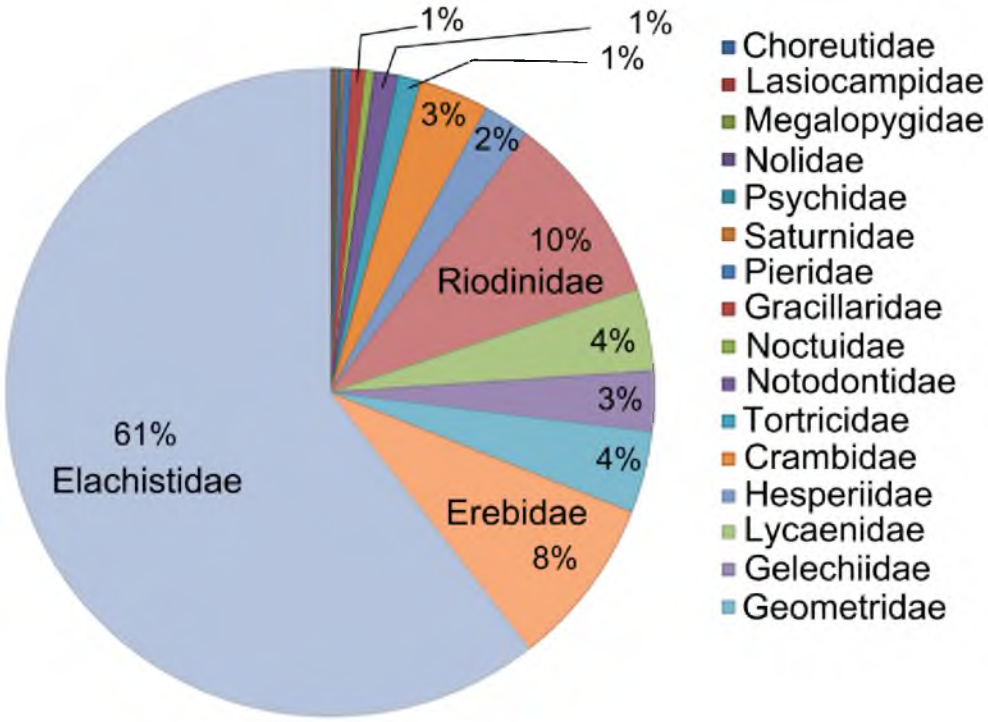
Appendix A.8. Ordination diagram of 31 *Inga capitata* plants based on similarities on their herbivore faunas (stress=0.01), with a cutoff number of 3 individuals per herbivore MOTU. Circles represent cap1, triangles represent cap2, and squares represent cap3.

Appendix A.9. DIC (Deviance Information Criterion) comparison of unconstrained vs. constrained models estimating the strength of preference of the sawflies for a particular ESU. More negative DIC values mean a better fit.

Model	DIC
Individuals constrained to have equal preference for the different ESUs (Constrained model)	-34.0
Variation in preference among ESUs (Unconstrained model)	-104.7

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 4



Appendix B.1. Lepidopteran herbivore families associated with *Inga* in Los Amigos. Percentages represent the relative abundances of the most abundant families. Elachistidae, Erebidae and Riodinidae are the families that were analyzed separately with and without joint-absence information.

Appendix B.2. Detailed methods

Leaf defensive traits were collected from young leaves on 0.5-4 m tall saplings in the shaded understory between 2007 and 2011. Leaf expansion rate was determined by measuring the area of leaves between 20% and 80% of full size every 1-4 days until they were fully expanded. Chlorophyll content of leaves between 30 to 80% of full expansion was estimated using three values from a Minolta SPAD 502DL meter (Spectrum Technologies, Plainfield, IL, USA). For calibration between SPAD units and chlorophyll content, a portable Spectronic 20 (Milton Roy, NY, 119 USA) was first calibrated in the laboratory using expanding leaves. These were extracted with 90% acetone/10% water (v/v) containing Na2CO3, and centrifuged at 10,000 x g at 5°C. Absorbances were obtained using a narrow bandpass spectrometer at 647 and 664 nm. Chlorophyll content was determined using the equations of Jeffrey & Humphrey (1975). The same leaves were extracted in 95% ethanol containing Na2CO3, centrifuged at 10,000g at 5°C, and transmittance measured at 663 nm and 725 nm. Regression analysis gave the following equation for the portable spectrophotometer:

$$\text{Chl a + Chl b (mg m-2)} = \frac{152.4 * (A663-A725) (\text{volume in mL})}{127 \text{ mL} * (\text{area in mm2}) \; 128} \qquad (1)$$

where Chl a + Chl b, is the total content of chlorophyll a and b; A663 and A725 are the absorbance readings for wavelengths of 663 nm and 725 nm, respectively.

In the field, the SPAD meter was calibrated using expanding leaves. The SPAD meter was used according to the manufacturer's directions. For determination with the

Spectronic 20, chlorophyll was extracted in 95% ethanol containing a small amount of NaHCO3, Na2CO3, or Na2HPO4 and centrifuged at 25 °C in a mini-centrifuge (SC1006-R, Roebling, NJ) at 2,000 x g. The relationship was non-linear and the equation to convert SPAD units to chlorophyll in mg of chlorophyll *a* and *b* m^{-2} is:

$$Chl=\alpha*SPADi^{\beta} \quad (2)$$

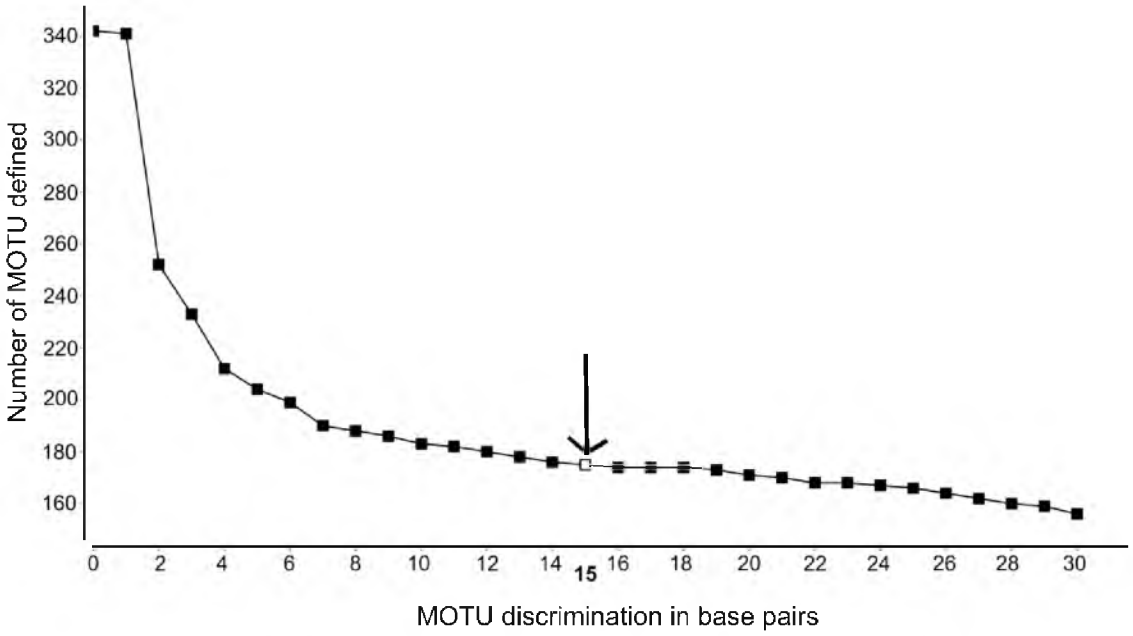
where Chl is the total content of chlorophyll (*a* and *b*) of the sample *i*, SPAD is the unitless reading from the SPAD 502DL meter, and α (0.0417) and β (0.9524) are the fitted parameters.

Timing and synchrony in leaf production were estimated by marking between 30-70 individuals per species and monitoring each plant monthly for the presence of young leaves. To estimate the timing of leaf production, or *mean angle,* we converted months to angles, from 0°= January to 360°=December at intervals of 30°. The *mean angle* for a species indicates the average date of peak flushing activity among the individuals. We evaluated the significance of the *mean angle* using the *Rayleigh test* (Zar 1999). We estimated synchrony in leaf production by calculating the coefficient of variation (cv) of plant individuals per species flushing each month.

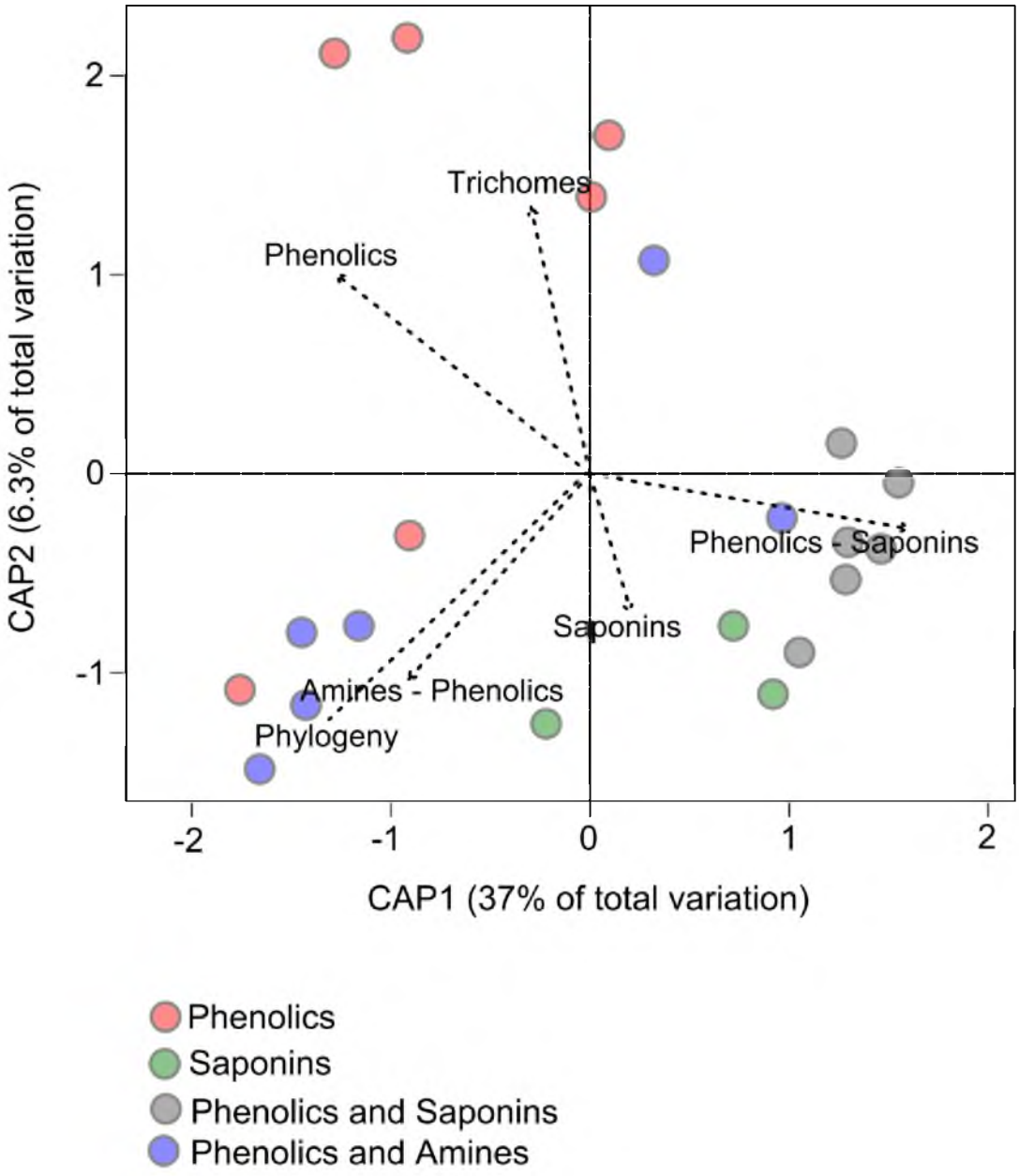
We also determined the number and identity of ants visiting the extra-floral nectaries during leaf expansion. Ants were identified to genus, and in some cases to species based on morphology by Dr. D. Donoso, an expert on the systematics of Neotropical rainforest ants.

Appendix B.3. PCR protocol

Reagents	Volume (ul) in standard PCR reactions
Multiplex Master Mix (Qiagen)	10
Water	6.6
Q-solution (Qiagen)	2
Forward 1 (20uM)	0.2
Reverse 2 (20uM)	0.2
DNA template	1
Total	20



Appendix B.4. Number of MOTU (Molecular Taxonomic Operational Unit) defined at each cutoff value. The arrow shows the cutoff used for this study (15 base pairs).



Appendix B.5. Constrained Analysis of PCO of the most parsimonious model for the lepidoptera community similarity measured by the Bray-Curtis index (R^2_{adj}= 0.42, p=0.001). This analysis included only species for which we had data on phenology of leaf production. Each dot represents an *Inga* species host color-coded by defense chemistry.

References

Jeffrey, S.W. & Humphrey, G.F. (1975) New spectrophotometric equations for determining chlorophyll a, b, c1 and c2 in higher plants and natural phytoplankton. *Bioch Physiol Pflanz (BPP),* **165**:191–194.

Zar, J. H. (1999) *Biostatistical Analysis*. Prentice-Hall, Upper Saddle River, New Jersey.